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INGRID MORING CLAESSON

The effect of ultraviolet light (λ 2537 Å) on *Helix pomatia* hemocyanin and bovine serum albumin as studied by the changes in the ultraviolet spectrum and sedimentation behaviour

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The effect of ultraviolet light (λ 2537 Å) on *Helix pomatia* hemocyanin and bovine serum albumin as studied by the changes in the ultraviolet spectrum and sedimentation behaviour

INAUGURAL DISSERTATION

BY

INGRID MORING CLAESSON
FIL. LIC., VÄRML.

BY DUE PERMISSION OF THE PHILOSOPHICAL FACULTY
OF THE UNIVERSITY OF UPPSALA,
TO BE PUBLICLY DISCUSSED IN THE LECTURE HALL OF
THE INSTITUTE OF CHEMISTRY, MAY 28, 1956, AT 10 A.M.,
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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The effect of ultraviolet light (λ 2537 Å) on *Helix pomatia* hemocyanin and bovine serum albumin as studied by the changes in the ultraviolet spectrum and sedimentation behaviour

By Ingrid Moring Claesson

With 64 figures in the text

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Preface

The present investigation was carried out at the Institute of Physical Chemistry of the University of Uppsala during the years 1951–1955.

To the Head of the Institute, Professor Stig Claesson, who is also my husband, I want to express my sincere gratitude for the facilities placed at my disposal, for the many helpful discussions, and for all the encouragement I have received.

To Professor The Svedberg, Director of The Gustaf Werner Institute for Nuclear Chemistry, I am also greatly indebted. His kind interest in the present investigation, in a field where he is the initiator, has been of great value to me, not to mention his personal friendship throughout the years.

Thanks are also due to my friend Docent Sven Brohult, LKB Laboratory, Äppelviken (Stockholm), who was my first teacher in physical chemistry. It has been a great pleasure and privilege for me to continue some of his work.

During his stay in Uppsala in 1950 Professor Douglas McLaren, University of California, Berkeley, who has performed so much pioneer work in the photochemistry of proteins, inspired the present work by his great enthusiasm. I wish to thank him for his advice and friendship.

The routine calculations in connection with the ultracentrifugations have been performed by members of the Calculation Department of the Institute. I wish to express my thanks to them, and especially to Miss Anna-Lisa Norling for her careful work.

For many years Mrs. Ingrid Öberg has been my technical assistant. Her thorough and skilful work has been of very great help to me, and it is a pleasure for me to thank her most cordially here.

The English text has been revised by Dr. Jim Manley and I am thankful for his many suggestions.

Uppsala, April 1956

Introduction

Although the effects of sunlight on proteins had been observed for a long time it was not until after the discovery of X-rays and radioactivity and after the development of the quantum theory that actual research was started on the irradiation of proteins.

The action of ultraviolet light as well as of visible light acting through sensitizers was observed and compared with that of more energy-rich radiations. It was soon realized that denaturation occurred and the time and conditions required for visible coagulum to form was one of the main lines of research. Many results had a qualitative rather than quantitative character. Changes in ultraviolet absorption spectra, viscosity, optical rotation, surface tension, electrical conductivity, refraction of light etc. were observed and in some cases the effect of pH and salt concentration noted. The results of various investigators could, however, seldom be compared as the experimental conditions were in general quite different. Arnow (1) has reviewed the results obtained up to 1936.

However, with the development of better physico-chemical methods and above all with the coming into use of the new sources of high energy radiation, e.g. accelerators and atomic reactors, the interest in the ionizing irradiation of proteins, peptides and amino acids has increased considerably. Two recent monographs, one by Bacq and Alexander (2) and one edited by Hollaender (3) deal with this subject.

Parallel with the investigations with high-energy irradiation, photochemical investigations have also been performed in increasing number. In a comprehensive review (4) McLaren gives a rather detailed account of the photochemistry of proteins and related substances up to 1949. Valuable information may also be found in volume II of the monograph edited by Hollaender and in a review by Doty and Geiduschek (5). Though much work has been done it is quite evident that the research in this field is in its beginning. Already the photochemistry of molecules with only a few atoms has proved to be far from simple and it is thus not surprising if that of protein solutions, often in buffer systems, presents a tremendously more complicated picture.

That protein molecules, after having absorbed light of sufficiently high energy, may show changes in almost all their physico-chemical properties is to be expected. Many workers have investigated proteins that show some specific activity, for instance an enzyme or a hormone, and measured the loss of activity as a function of

the number of absorbed light quanta (4, 6, 7, 8). It is often impossible, however, to decide if the loss is due to the direct action of the light on a specific group or if it is brought about as a result of splitting or aggregation of the molecules or a combination thereof. In general, one can state that knowledge of changes in the molecular weight is desirable before conclusions can be drawn from measurements of any other changed property.

The present investigation, performed during 1951–1955, deals with some of the effects of ultraviolet light ($\lambda = 2537$ Å) on two proteins, *Helix pomatia* hemocyanin and bovine serum albumin. The author has been principally interested in the changes in the molecular weight. It has also been necessary to study the ultraviolet spectra and in this connection some amino acids and peptides were also investigated.

Ultracentrifugal studies of irradiated protein solutions were started in the 1930's. Pedersen (9) and Sanigar *et al.* (10) were using serum albumin, Svedberg and Brohult (11) and later Brohult (12) investigated hemocyanin and serum albumin. Since then irradiated bovine serum albumin has been studied for instance by osmotic pressure measurements by Rideal and Roberts (13). Spectral changes after irradiation have been noticed by a great many workers for amino acids, peptides and proteins (4). Special references will be given in connection with the presentation of the experimental data in chapter III.

The irradiation of proteins with ultraviolet light may of course be performed under different experimental conditions. The author has chosen to work with aqueous solutions, often buffered, and in an atmosphere of air, oxygen or nitrogen. The reason for this is that it seemed preferable to work under conditions that are fairly similar to those that exist in nature and similar also to those used by most of the earlier workers in this field. It is true that the solvent molecules as well as the dissolved gas molecules complicate the reaction, but irradiation of dry proteins gives very small amounts of changed material and can only be used in special cases, e.g. when changes in enzyme activity are studied (14). Monolayers of proteins have been irradiated (15,16) and the results are specially interesting as the molecules are oriented in a specific way; here also the amount of material will be small.

There are at present no generally applicable methods for fractionating very complicated protein mixtures though some chromatographic methods show great promise (17, 18). The investigation has therefore been made on the entire protein mixture resulting from the irradiation. Under such circumstances measurements for example of osmotic pressure and light scattering which give an average molecular weight without telling anything about the molecular weight distribution are less suited for the problem than measurements with the ultracentrifuge. Since the latter method tells whether splitting or aggregation has occurred and also gives the concentration of the unchanged molecules it has been almost exclusively used in this investigation.

The literature on the photochemistry of proteins, amino acids and peptides is very rich and in order not to make the number of references unduly large the author has often had to adopt the rule of only giving references to fairly recent papers and to review articles.

Chapter I

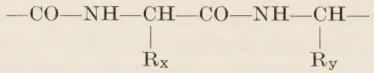
Ultraviolet spectra of unirradiated substances containing the protein chromophores

It has long been known that only the light that is absorbed by a substance is able to change it photochemically (Grotthus 1817, Draper 1841). Therefore in studying the action of light on a substance the first task is to determine its absorption spectrum.

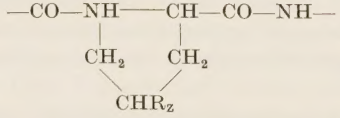
Another important thing is to find out whether the light absorbed has enough energy to bring about a change in the molecule, i.e. to break a bond. Bond strengths have almost entirely been determined for bonds between two atoms in a molecule with few atoms and as the bond strengths vary with different substituents the existing data will only approximately hold for similar bonds in a large molecule (19, 20). By using the normal values for the bond strength it is estimated (4) that it is possible for any single bond in a protein molecule to be broken by light of energy higher than 100 kcal/einstein, that is for wavelengths shorter than 2850 Å.

Proteins show no absorption for wavelengths longer than 3000 Å. In the few cases of coloured proteins the absorption[1] is caused by some specific group, containing for instance a metal atom, and is not characteristic of the protein as such. Since a detailed description of the ultraviolet spectrum of proteins has recently been given by Beaven and Holiday (21) and Dannenberg (22) and also by Doty and Geiduschek (5) only the most important facts will be given here.

A protein molecule is built up of one or several peptide chains, that is by amino acids connected with each other by the so called "peptide bond" –CO–NH– resulting from the reaction of a carboxyl group in one acid with the amino group (generally in the α-position) in another. The pattern



will recur throughout the whole molecule, R_x and R_y being the characteristic substituents of the amino acids. Only in the case of proline and oxyproline which are the only amino acids that cannot be described as a substituted glycine, does a different grouping exist, namely



where R_z = H for proline and OH for oxyproline.

Investigations by Pauling and Corey (23) have shown that the peptide chain or chains are present in the form of helices, and that hydrogen bonds existing both between groups in the same chain and between groups in the neighbouring chains stabilize the structure. Furthermore, the existence of –S–S– bonds between chains also plays a role in stabilizing the structure.

[1] Mostly in the visible.

The spectrum of a protein will thus be determined by the light absorption of the chromophores in the sidechains of the constituent amino acids, the peptide bond absorption, the –S–S– bond absorption, possible additional structural absorption and light scattering due to the size of the protein molecule.

For the determination of the extinction coefficient E we have the well-known Beer's law

$$\log \frac{I_0}{I} = E \times c \times l = d$$

where I_0 is the intensity of the incident light, I that of the transmitted, c the concentration and l the thickness of the layer that the light has passed and d the optical density. In this investigation the symbol E is the extinction coefficient for $c = 1$ % and $l = 1$ cm. For $c = 1$ mol/lit and $l = 1$ cm the symbol ε, the molar extinction coefficient, is used. The optical density is measured with $l = 1$ cm.

When both light absorption and light scattering are present the extinction coefficient E is the sum of the absorption coefficient E_a and the scattering coefficient E_s.

Light absorption, general

In the region 3000–2500 Å the absorption of a protein is essentially that of a mixture of the constituent amino acids and is primarily caused by the aromatic ones and cystine where the aromatic groups and the –S–S– bond are the absorbers (21).

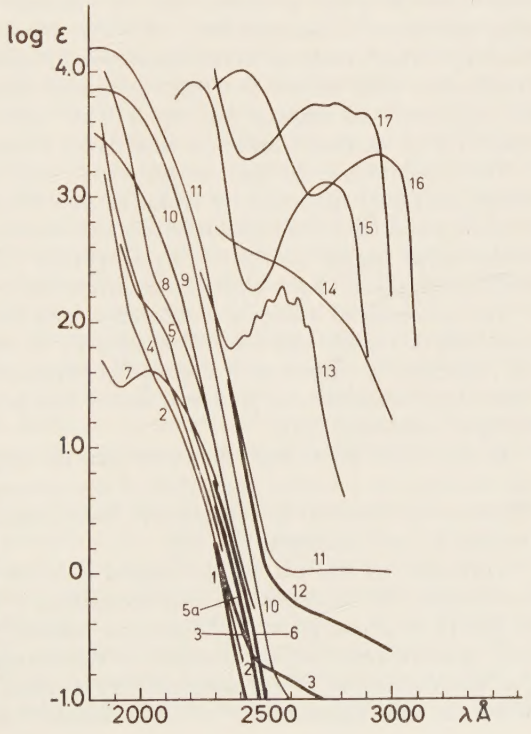


Fig. 1. Absorption spectra of **1** glycine, **2** glycine according to (28), **3** valine, **4** valine (28), **5a** sodium acetate, **5** sodium acetate (28), **6** acetic acid, **7** acetic acid (28), **8** acetamide (28), **9** dietylamine (28), **10** diglycine (24), **11** triglycine (24). **12** triglycine, **13** phenylalanine, **14** cystine in 0.1*N* HCl (21), **15** tyrosine (21), **16** tyrosine in 0.1*N* NaOH (21), **17** tryptophan (21). If not otherwise stated the solvent is H_2O. Heavy lines are own measurements.

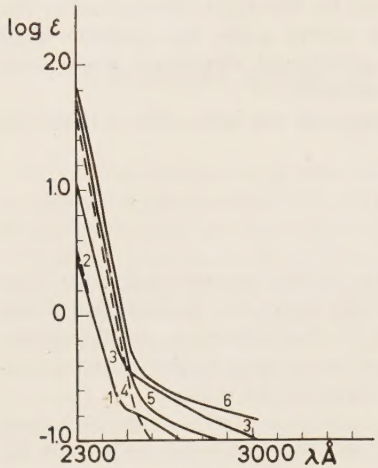


Fig. 2. Absorption spectra of valine: **1** in H_2O, **2** according to (28), **3** in 0.1*N* NaOH; and of diglycine: **4** according to (24), **5** in H_2O, **6** in 0.1*N* NaOH.

However, when the amino acids are incorporated in the protein the absorption bands of tyrosine and tryptophan have moved 15–30 Å, and those of phenylalanine up to 10 Å towards longer wavelengths (21). In lower peptides the changes are only about 4–9 Å (21). Figs. 1, 4, and 6 show some relevant spectra.

That the peptide bond should show absorption of any importance in the region 3000–2500 Å is not probable (21, 24). Recent findings by Liquori *et al.* (25) show that polymers containing the –CO–NH– bond develop appreciable absorption after heating—which remains in the hydrolyzed product—with a maximum around 2900 Å. Irradiation with ultraviolet light produces changes of seemingly the same kind on leucylglycine and clupein, see Fig. 3. The "peptide bond absorption" around 2800 Å observed by various workers is thus quite likely due to impurities.

The contribution of the non-aromatic acid in this region is also negligible, see below, and the suggestion by McLaren and Waldt (26) that the appreciable difference in absorption between chymotrypsin and its aromatic acids is due to the other amino acids seems highly unlikely. Chymotrypsin has a high tryptophan content and a small error there might explain the difference.

The presence of additional structural absorption in the protein in the mentioned wavelength region has been discussed by a number of workers (21). Schauenstein (27) reports the existence of a so-called peptenol absorption with a maximum around 2500 Å in some fibrous proteins, but it has been questioned if the effect is not due to light scattering (21).

In the region 2500–1800 Å, where also the aliphatic amino acids absorb (28), Fig. 1, the absorption is higher than that of the corresponding amino acid mixture and the difference is ascribed to the peptide bond (maximum around 1850 Å) which may be present in the enol form (24, 29).

Variation of the pH in the region 2–8 leaves the protein spectrum practically unchanged, but at higher pH's the ionization of the hydroxyl group of tyrosine starts, at pH 12 it is complete, and causes a marked shift towards longer wavelengths, as well as an increase in the intensity of the maximum. This has actually been used for the determination of tyrosine and tryptophan in a protein from the spectrum (21). However, the applicability of this method has been questioned since it has been

observed by Crammer and Neuberger (30) that in certain proteins complete ionization does not occur until pH 13 where the protein is denatured and those hydrogen bonds, which evidently prevented the ionization of the hydroxyl group, are broken. Measurements at pH 13 are sometimes difficult as the spectrum may change with time. We have here, however, a method to determine the free tyrosine groups in the protein.

The protein spectra are not sensitive to small temperature changes around room temperature but at very low temperatures there are distinct differences compared with spectra at room temperature. The fine structure is very clearly seen at 77°K and from this valuable information has been derived by Loofbourow *et al.* (31).

Light absorption at λ 2537 Å

As the wavelength 2537 Å has been used in the present investigation it is relevant to state in what groups this radiation is absorbed. All the aromatic groups and the –S–S– bonds absorb here, see Fig. 1, whereas the side chains of the non-aromatic amino acids show rather slight absorption, see below. It has also been much discussed whether the -CO–NH– bond has any absorption (21). On theoretical grounds this is possible but it must be rather low (24). The interest in the peptide bond absorption at 2537 Å has been especially great as it is generally believed that splitting upon irradiation occurs in this bond.

The peptide bond absorption at low wavelengths (1850–2100 Å) has been determined by Ham and Platt (24) from the difference in the absorption of triglycine and diglycine and by Goldfarb, Saidel and Mosovich (29) from the difference between the absorption of a protein and its constituent amino acids as well as from the difference between the absorption of some aliphatic peptides and the corresponding amino acids. Near 2537 Å, where only very small differences are to be expected, the uncertainty will be great and very different estimations of the peptide bond absorption exist. From the values of Saidel and Goldfarb in the paper by Ham and Platt (see Figs. 1 and 2) the molar extinction coefficient of diglycine is 0.17. Setlow and Guild (32) find a value of about 2.4 for the molar absorption of the peptide bond from measurements of leucylglycine, leucine and glycine. This high value is probably due to impurities as a rather high absorption was also reported at 2800 Å. McLaren and Waldt (26) take the difference between the molar absorption of acetylalanine $\varepsilon = 1.28$ and acetic acid $\varepsilon = 0.02$ and get $\varepsilon = 1.26$. Rideal and Roberts (13) estimate rather generally that about 5 % of the total absorption of a protein at 2537 Å is due to the peptide bond. This value was derived from the differences in the spectra of proteins and their constituent amino acids. However, due to the wavelength shift for the incorporated aromatic amino acids and the fact that a slightly too low value of the tryptophan content (which is difficult to determine accurately) in the amino acid mixture will appreciably lower the absorption, such calculations may give rather unreliable results.

The following reasoning will also show that it is difficult to measure the "peptide bond absorption" by comparing a protein with its hydrolysate even if we did not have to consider the above-mentioned wavelength shift. Suppose that we have a polypeptide containing n monomer units each with the molecular weight, M. The number of peptide bonds will then be $n - 1$. A solution with a given concentration of a g/l has then $a(n-1)/nM$ peptide bonds. The molar absorption of the latter is assumed to be x. For $n = 2$ the "peptide bond absorption" is $xa/2M$, for large n it is xa/M. Accordingly, in solutions of the same concentration of a poly-

peptide and a dipeptide respectively, the first has only twice the absorption of the latter. This is also immediately clear from the fact that one amino acid can at most provide two peptide bonds in a polypeptide, in a dipeptide only one bond is present. During hydrolysis of a protein resulting in for instance decapeptides, the "peptide bond" absorption and consequently the total optical density decreases from xa/M to $0.9xa/M$. For $M = 150$ and $a = 1$ (at 2537 Å the optical density at this concentration is normally around 0.5–0.6) the difference is $0.1\ x/150$ which for x in the order of 1 has a value of the order of 0.001 which is too low to be noticed in the Beckmann spectrophotometer. The total "peptide bond absorption" will be less than 0.01.

Measurements of the peptide bond absorption

The present author has determined the spectra of diglycine, leucylglycine and triglycine and for comparison also those of glycine and valine in the region 2300–3000 Å in order to get an estimate of the peptide bond absorption here. Arginine was determined for comparison with clupein, see below. The results will also show the degree of accuracy obtained at the light absorption measurements in the present investigation.

The amino acids and peptides investigated by the present author were of good commercial quality (Merck and Hoffmann-La Roche) and used without further purification. No traces of other amino acids and peptides were observed when examined by paper chromatography. They were dissolved in distilled water, filtered through a bacterial filter and run in a Spinco centrifuge to get rid of optical impurities. The concentrations used were 0.1–0.05 molar except for the lower wavelengths where the solutions were diluted to give an optical density around 0.5.

The measurements of the spectra were made with a model DU Beckmann spectrophotometer, and reliable data could be obtained down to approximately 2300 Å. At lower wavelengths irregularities of well known type began to appear (33). The results are given in Figs. 1, 2, and 3.

Ley and Arends (28) have measured the spectra of very pure aliphatic amino acids, fatty acids, amines and amides, see Fig. 1. They found that glycine (α-aminoacetic acid) does not show a shift towards the red in comparison with acetic acid as one would expect from the influence of the amino group. The ampholytic character instead causes a shift towards shorter wavelengths. In strong acid the absorption is nearly the same as that for acetic acid and in strong alkali the shift towards longer wavelengths is even somewhat larger due to the amino group. Increased chain length only causes a slight shift towards longer wavelengths; leucine, valine, proline, oxyproline and piperido acetic acid have nearly identical spectra. Threonine, serine, isoleucine also show the same absorption spectrum as valine, Saidel *et al.* (34) have determined the spectra in the region 2100–2300 Å.

It is seen that the curves for valine and glycine only differ very little from those of Ley and Arends, the very slight absorption for $\lambda > 2500$ is undoubtly due to very small amounts of impurities.

It is clear that valine has a molar extinction coefficient of less than 0.1 for 2537 Å. The same will apply to leucine, proline, oxyproline, threonine, isoleucine and serine. Arginine also shows a similar low absorption (see Fig. 3). Aspartic acid and glutamic acid have an ε value of the order of 1 (35) and for histidine $\varepsilon \sim 5$ (36). The values reported for methionine and cysteine are $\varepsilon \sim 1$ and $\varepsilon \sim 7$ resp. (21). It is then evident that in a normal protein holding approximately 10–15 % of the strongly absorbing

amino acids the contribution at 2537 Å of the other amino acids to the absorption will be of the order of 1 %.

The curve for diglycine, Fig. 2, falls very close to that given by Saidel and Goldfarb in reference (24) whereas their triglycine probably contains some impurities. Valine and diglycine only differ by the presence of the peptide bond in the latter and a different distance between the ionized groups and it is interesting to compare the curves. The molar extinction coefficient for diglycine, both in neutral and in strongly alkaline media, is lower than that reported for acetylalanine $\varepsilon = 1.28$ (26) which suggests that the latter value may be too high. It is seen that the molar peptide absorption for the simplest peptides with no sidechains is of the order of 0.1.

It has, however, been observed that sidechains will increase the peptide bond absorption at lower wavelengths, in proteins a larger value of the average peptide bond absorption is also found (29). Clupein which is a polypeptide (the molecular weight is generally given as 3000–4000) containing only aliphatic amino acids (arginine, approx. 70 %, alanine, serine, proline and valine and small amounts of threonine and isoleucine (37)) is well suited for the study of the peptide bond absorption in a protein, though it may be argued (21) that the influence of surrounding groups is not as fully developed as in a protein itself. The spectrum has been investigated by McLaren (26, 38) and also by Beaven *et al.* referred to in (21), and they conclude that the peptide bond absorption at $\lambda = 2537$ Å must be small.

The present author determined the spectrum of a sample of clupein sulfate from the Carlsberg Laboratories, which also provided McLaren with his material. Great care was taken to remove optical impurities. This was accomplished by a combination of filtration and high-speed centrifugation. The solvent was a dilute phosphate buffer at pH 6.2 and the concentration counted on the clupein (the sulfate contains 80 % clupein (39)) was between 0.8 % and 1.3 % for measurements in the region 4000–2400 Å; for lower wavelengths suitable dilutions were used. The molar absorption of the average amino acid residue in clupein (residue weight 137 calculated from amino acid values given by Felix *et al.* (37)) is seen in Fig. 3. Values calculated from the curve of McLaren are also plotted and the agreement is surprisingly good. That the flat absorption in the region 2500–3000 Å would be entirely due to light scattering as suggested by Beaven and Holiday (21) seems rather unlikely. As mentioned before, arginine as well as the other constituent amino acids shows negligible absorption. Either the large side chains have influenced the peptide bond absorption or some other groups or material must be responsible.

If we consider the value derived from clupein as an upper limit of the molar peptide bond absorption we get $\varepsilon < 1.74$. For practical purposes ε may then be considered

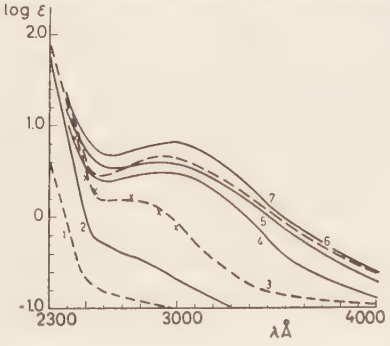


Fig. 3. Absorption spectra of **1** arginine, **2** leucylglycine, **3** clupein in phosphate buffer at pH 6.2, × corresponds to values from (38), **4**, **5**, **7** leucylglycine irradiated with $Q/d_{2537} = 4700$, 14,000 and 65,000 × 10^{18} $h\nu$/ml resp. and with $d_{2537} = 0.05$, **6** clupein in phosphate buffer at pH 6.2 irradiated with $Q/d_{2537} = 400 \times 10^{18}$ $h\nu$/ml with $d_{2537} = 0.13$. If not otherwise stated the solvent is H_2O. For explanation of Q/d_{2537} see chapter III.

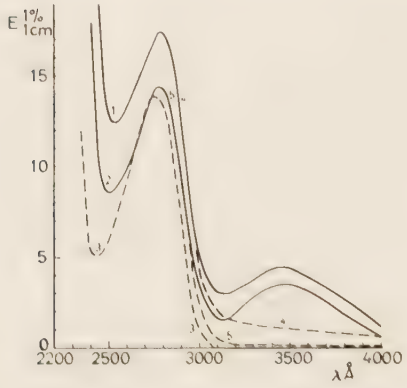


Fig. 4. Absorption spectra of *Helix pomatia* hemocyanin. 1 Undissociated hemocyanin, oxidized form, 2 hemocyanin dissociated into eighths, oxidized form, 3 corresponding aromatic amino acid mixture at pH 6.2, 4 undissociated hemocyanin, reduced form, 5 hemocyanin dissociated into eighths, copper free form. Curves 4 and 5 are identical with 1 and 2 for wavelengths shorter than 3000 Å.

to be of the order of 1 at 2537 Å, an estimation also given in (21). As to whether this absorption will constitute a considerable part of the total absorption of a protein is naturally dependent on the amount of strongly absorbing amino acids. Gelatine which for a chosen molecular weight of 100,000 contains approximately 1000 peptide bonds would thus have an absorption of 1000 from the peptide bond. The absorption from the phenylalanine and tyrosine in the molecule will be approximately 6000 and thus the peptide bond absorption becomes important whereas for serum albumin the molar absorption is around 20,000 and the peptide bond absorption about 600.

Light scattering

The effects of light scattering must be taken into consideration in evaluating protein spectra.

The absorption coefficient E_a and the scattering coefficient E_s are additive and their sum is the extinction coefficient E calculated from the spectrum by means of Beer's law. Treiber and Schauenstein (40) have verified this for a number of proteins both for two component systems where one component showed only scattering, the other only absorption and for systems where the same molecules showed both scattering and absorption. According to Rayleigh and Mie the scattering coefficient $E_s = K\lambda^{-n}$, where λ is the wavelength and K and n constants; it then follows

$$\frac{d \log E_s}{d \log \frac{1}{\lambda}} = n.$$

Schauenstein and Treiber plotted log E against log λ and found constant values for K and n not only in the wavelength region where $E = E_s$ but also for 3000–2500 Å where the absorption is appreciable for proteins. The value of n, however, varies with the size and shape of the molecules and $n = 4$ given in the original Rayleigh formula is usually only valid for molecules with a length < 1/10 of the wavelength. For larger molecules the n values decrease and for very large particles the scattering coefficient is independent of the wavelength. With a freshly prepared gelatine solution the above authors found $n = 4$, on standing the same solution showed $n = 2.5$, indicating the formation of larger aggregates. Schauenstein and Bayzer (41) have

given new evidence for the constancy of K and n from measurements on γ-globulin in non-aggregated and partly aggregated form. The n values are between 2 and 3. Schramm and Dannenberg (42) had earlier found that K and n were constant for tobacco mosaic virus with $n = 4$, even for the wavelength region where the absorption is large, they only made use of the two wavelengths 3130 Å and 4050 Å for their calculation of n. Doty and Geiduschek (5) discuss the different ways of correcting protein absorption spectra for light scattering and point out that the constancy of K in the absorption region is not absolutely certain in all cases: the absorption coefficient E_a from the corrected spectrum may only be qualitatively correct.

In summarizing, it may be pointed out that even after making allowances for light scattering it is not surprising if the absorption of a protein is not completely accounted for by the constituent amino acids determined by the usual analysis after hydrolysis. Losses may easily have occurred and especially the value for the tryptophan content has a great influence. The difficulty of making correct tryptophan analyses has been pointed out by Tristram (43). Decomposition products of the acids present in the protein already before hydrolysis may also be responsible for some of the "extra" absorption. It is thus clear that too far-reaching conclusions of the composition and structure of a protein cannot be drawn from evidence resting entirely on the appearance of the absorption spectrum.

Helix pomatia hemocyanin

References to earlier investigations of the hemocyanin spectrum are given by Dawson and Mallette (44) and in (45).

Helix pomatia hemocyanin is a respiratory protein, where the oxygen is combined with copper. The oxidized and reduced forms have the same spectrum for wavelengths below 3000 Å with a maximum at 2780 Å (the protein band). Above 3000 Å oxidized hemocyanin shows two bands one at 3460 Å and another in the visible at 5570 Å both due to the oxidized copper groups. The exact way in which the oxygen is bound to the copper is a matter under discussion (46, 47). The ratio between oxygen and copper atoms is established as 1 (44) and it seems likely that the copper is bound to sulfur (46) but also co-ordinated with an amino group (48).

For undissociated hemocyanin ($M = 9 \times 10^6$) considerable light scattering is observed. Lontie *et al.* (45) have investigated the influence of light scattering on the extinction coefficient E of this protein at 2780 Å (the protein band) and 3460 Å (the copper band). As hemocyanin dissociates into subunits when the buffer and pH are suitably changed the light scattering is accordingly diminished, see chapter VI. Lontie *et al.* measured the extinction coefficient at 2780 Å and 3460 Å and the specific scattering power at 5780 Å at an angle of 90° for solutions exhibiting different states of dissociation. The ordinates of the specific scattering power and of the extinction coefficient at the chosen wavelength were adjusted to each other so that the maximum values coincided. The zero value of the specific scattering power should then coincide with a value for the extinction coefficient answering to $E_\lambda = 0$, that is, to the absorption coefficient E_a. The values are found in Table 1.

In the present investigation the spectra of a number of hemocyanin preparations were determined. Description of the material is found in chapter VI. The amino acid composition of hemocyanin is seen in Table 2. The determinations were per-

Table 1. The extinction and absorption coefficients E at certain wavelengths of *Helix pomatia* hemocyanin and bovine serum albumin.

Substance		2537 Å	2780 Å	3460 Å	4000 Å	5570 Å
Hemocyanin component I (wholes) $M = 9 \times 10^6$ pH = 6.2	E^{ox}	12.4	17.4 17.6 R 17.05 L	4.41 4.63 R 3.80 L	1.10 1.32 R	0.35
	E^{red}	12.5	17.4	1.2 1.8 R	0.56 0.71 R	0.15
	E_s	3.5*	2.3* 3.22 L	1.0* 0.90 L	0.56*	0.15*
	E_a^{ox}	8.9**	15.1** 13.83 L	3.4 2.90 L	0.54	0.22
	E_a^{red}	8.9**	15.1**	0.2	0	0
Hemocyanin component III (eighths) $M = 1 \times 10^6$ pH = 8.2	E^{ox}	8.7	14.4	3.52	0.66	0.23
	E^{red}	8.7	14.4	0.23	0.13	0.03
	E_s	0.72*	0.50*	0.21*	0.12*	0.03*
	E_a^{ox}	8.0	13.9	3.31	0.54	0.20
	E_a^{red}	8.0	13.9	0.02	0.01	0
Bovine serum albumin $M = 69.000$ pH = 6.2	E	3.03	6.54			

E^{ox} and E^{red} are the extinction coefficients of the oxidized and reduced hemocyanin resp. $E_a = E - E_s$. On account of the difficulties of removing the oxygen at pH 8.2 without denaturation occurring when passing nitrogen through the solution, measurements were instead made on the copper free form at this pH. The copper was removed by KCN according to Kubowitz (52).

* Values corresponding to $n = 4$ in Fig. 5.

** Values obviously too high.

R = Values from Roche and Dubouloz (50).

L = Values from Lontie *et al.* (45).

formed by the usual chromatographic methods.[1] Tryptophan and cystine were not determined but have been estimated. Tryptophan was estimated from the difference between the absorption coefficient of hemocyanin at 2780 Å and the absorption coefficients of a mixture of phenylalanine and tyrosine in proportions according to values in Table 2. Cystine was estimated from the difference between the total sulfur content and that of the methionine content in the table. The sum of the amino acids is rather low. A value about 10 % higher would have been expected. Values from determinations by Roche and Jean in (44) are given for comparison.

The concentration of the hemocyanin solution was determined refractometrically on a concentrated stock solution that then was diluted suitably for the measurements of the spectrum. The refractive index increment 186.3×10^{-5} at 5460 Å was used at all pH's (49).

Before the measurements of the spectra the solutions were cleaned by centrifugation at 3500 r.p.m. for one hour as recommended by Lontie. Filtration through bacterial filters was also used at times.

Fig. 4 shows the spectra at pH 6.2 ($M = 9 \times 10^6$) and pH 8.2 ($M = 1 \times 10^6$) for a representative preparation. The spectrum of the corresponding aromatic amino

[1] The author is greatly indebted both to Prof. O. Mellander at the Laboratory of Medical Chemistry, University of Gothenburg, where the determinations were performed, and to Mr. Strid who carried out the experiments.

Table 2. Amino acid composition of unirradiated and irradiated *Helix pomatia* hemocyanin and of unirradiated bovine serum albumin.

Amino acid	Unirradiated hemocyanin				Irradiated hemocyanin $Q/d_{2537} = 7.0 \times 10^{18}$ $h\nu$/ml		Unirradiated hemocyanin, A	Unirradiated bovine serum albumin B	
	g/100 g protein	% N	Min. molecular weights	Number of residues	g/100 g protein	% N	g/100 g protein	g/100 g protein	Number of residues $M = 69{,}000$
Aspartic acid	11.8	1.247	24,800	22	12.1	1.275		10.91	65
Glutamic acid	9.82	0.935	25,500	17	13.04	1.240		16.5	78
Glycine	3.46	0.627	25,900	12	3.88	0.724		1.82	17
Alanine	5.12	0.805	24,400	14	5.30	0.835	3.82	6.25	48
Valine	6.18	0.738	24.700	5	5.18	0.620	7.64	5.92	35
Leucine	9.55	1.020	24,700	18	9.46	1.010	9.17	12.27	65
Isoleucine	4.72	0.504	25,000	9	4.48	0.468		2.61	14
Serine	4.90	0.653	25,700	12	4.96	0.662		4.23	28
Threonine	5.49	0.644	25,500	12	5.16	0.606		5.83	29
Proline	5.25	0.638	24,200	11	5.68	0.692		4.75	29
Methionine	1.14	0.111	26,200	2	0.887	0.083		0.81	4
Phenylalanine	6.90	0.586	23,900	10	6.87	0.583		6.59	24
Tyrosine	6.47	0.473	24,300	6	6.56	0.508	4.59	5.06	19
Histidine	6.52	1.764	23,700	10	6.36	1.725	5.81	4.0	18
Lysine	5.15	0.978	25,640	9	4.90	0.940	7.46	12.82	60
Arginine	6.20	1.995	25,200	9	5.37	1.727	5.26	5.90	23
Ammonia	1.726*	1.42			1.78*	1.465		0.78	
Tryptophan	3.5[1]	0.480*	23,200	4			5.90	0.68[3]	2
Cystine	1.93[2]	0.225*	24,800	2			2.05		
Half cystine								5.73	28
Cysteine								0.3	2
Total	104.10	15.138	447,340	184	100.187	15.173		112.98	588
Per cent nitrogen accounted for		99.5				99.7		100.9	

* Not included in the sum.

[1] Estimated from the difference between the absorption coefficient of the protein at 2780 Å see Table 1 and that of a mixture of tyrosine and phenylalanine corresponding to the values in the above table.

[2] Estimated from the difference between the total sulphur content 0.76 % and that of the methionine in the table. No distinction between cystine and cysteine has been made. It is reported (122) that freshly prepared hemocyanin contains 150 thiol groups per molecule.

[3] The value 0.58 has earlier been given and corresponds exactly to 2 residues for $M = 69{,}000$. This discrepancy may possibly be due to a misprint. However, as a lower molecular weight has recently been proposed, a larger tryptophan value would also be needed for 2 residues.

A. Value from Roche and Jean cited in (44).

B. Values from Tristram in The Proteins, ed. Neurath, H. and Bailey, K., Vol. I A, p. 215.

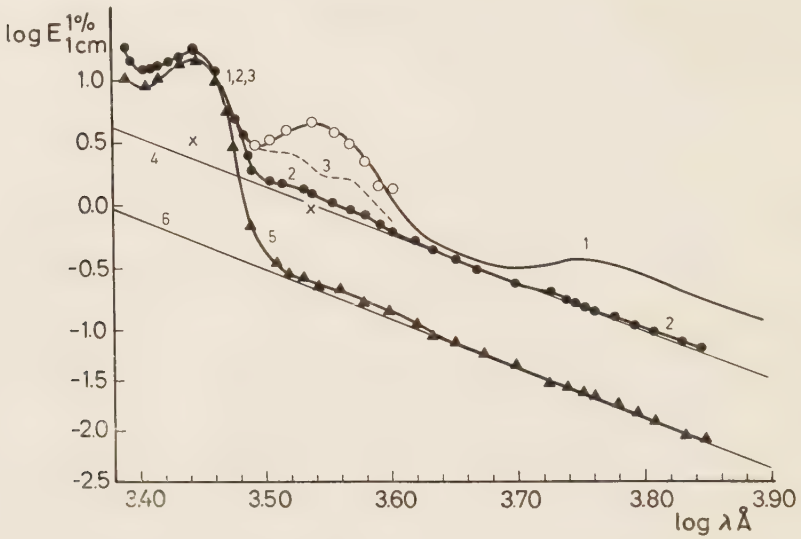


Fig. 5. Log $E^{1\,\%}_{1\,cm}$ plotted as a function of log λ. **1** Undissociated hemocyanin oxidized form, ○ are values from (50), **2** and **3** undissociated hemocyanin reduced form, **3** is the curve from ref. (50), **5** hemocyanin dissociated into eighths, copper-free form. **4** and **6** are straight lines with $n = 4$; × are points from (45). It has not seemed necessary to plot also the oxidized hemocyanin dissociated into eighths. The ultraviolet spectrum of the latter is seen in Fig. 4.

acid mixture, see Table 2, has also been plotted there. The extinction coefficients for λ 2300–3000 Å vary only slightly from preparation to preparation and are here the same for oxidized and reduced hemocyanin. These variations are no doubt due to small differences in the molecular weight distribution. Above 3000 Å larger differences may be encountered for the oxidized hemocyanin.

The oxygen combining capacity is thus not constant from preparation to preparation; even for the same preparation it decreases very slowly with time as already noticed by Roche and Dubouloz (50). In Fig. 5 log E has been plotted against log λ both for a solution of the oxidized hemocyanin at pH 6.2 showing the absorption bands at 3460 Å and 5560 Å, and for the reduced form. The latter form was obtained by passing a slow stream of nitrogen through the solution. This operation was carried out in the cuvette in the Beckmann spectrophotometer so that the measurements could be carried out immediately afterwards. It is seen that a straight line with $n = 4$ fits the points for the reduced form rather well above 3100 Å, in agreement with earlier measurements by Redfield (51) on other hemocyanins.

Around 3460 Å the observed values are somewhat too high, probably due to small amounts of oxidized hemocyanin. The discrepancies were, however, judged to be small enough to allow the use of nitrogen as the oxygen replacing agent, for the determinations in the wavelength region above 4000 Å they are of no importance. Incidentally Kubowitz (52) has noticed that carbon monoxide is a slightly more effective oxygen replace in this connection.

The corresponding values given by Roche and Dubouloz (50) have been plotted for comparison, using a nitrogen value of 15.15 % for the calculation of the concentration from their data. Their values at 3460 Å for the reduced form are rather high.

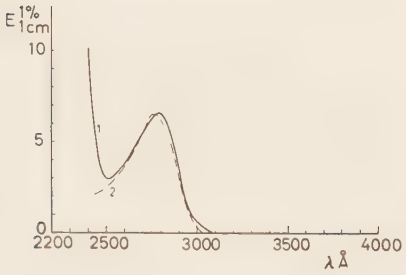


Fig. 6. Absorption spectra of 1 bovine serum albumin and 2 corresponding aromatic amino acid mixture in phosphate buffer pH 6.2.

For $\lambda = 2780$ Å the scattering coefficient, 0.322, determined by Lontie *et al.* is considerably higher than that answering to $n = 4$. In this case K is evidently not constant in the region of high absorption and the caution recommended by Doty and Geiduschek (5) thus proves to be well advised. Hemocyanin has a rather high absorption at λ 2780 Å, about twice that of bovine serum albumin for the same concentration and this may be significant in this connection. The spectrum of copperfree hemocyanin at pH 8.2 was also determined see Figs. 2 and 3. Also here $n = 4$ fits the points well above 3000 Å. The E_s values derived for $\lambda < 3000$ Å might be too low in analogy with the former case.

As the value of n is as high as 4 both for hemocyanin and for the still larger tobacco mosaic virus, it seems likely that aggregates of even larger size are responsible for the lower n value reported in (41).

The pertinent data are collected in Table 1.

It is important to get an appreciation of E_a and E_s for the undissociated hemocyanin; the value 8.0 for E_a measured at pH 8.2 was judged to be the best as the light-scattering correction was the smallest in this case. This value is somewhat higher than that of the corresponding aromatic amino acid mixture, see Fig. 3. The ratio E_s/E for undissociated hemocyanin is then 0.35.

The peptide bonds absorb 0.9 % of the total light absorbed, calculated with a mean residue weight of 136 and a molar absorption of 1 for the peptide bond.

Bovine serum albumin

Description of the serum albumin investigated is found in chapter VI. The amino acid composition is given in Table 2. The spectrum of serum albumin at pH 6.2 is seen in Fig. 6 where also the spectrum of the corresponding aromatic amino acid is plotted. The values for the latter is taken from Table 2 and fits better with the protein spectrum than when using a tryptophan value of 0.58 g/100 g protein (13), see further explanation in the table. The light scattering in the buffers used have been negligible. Values for the extinction coefficients at λ 2537 Å and 2780 Å are found in Table 1. The peptide bonds absorb 2.8 % of the total light absorbed, calculated with a mean residue weight of 117 and a molar absorption of 1 for the peptide bond.

Chapter II

Experimental arrangements for irradiation

General consideration

Very simple experimental arrangements were used. In principle it is only necessary to have a cuvette for the solution and a source of ultraviolet light. What has to be determined is the number of light quanta absorbed in the solution. A rather high intensity was desired in order to avoid irradiation times longer than a few hours and accordingly the light intensity was not diminished by parallelizing the light.

The amount of light absorbed by a solution of a given optical density in a cuvette through which a certain amount of non-parallel light passes is best determined from reference experiments where the solution in question is substituted by an actinometer solution of the same optical density and refractive index. The decomposition of the latter is then determined and the number of absorbed quanta calculated. Errors arising from reflections at the walls and entrance windows of the cuvette are eliminated in this way and the accuracy of the determination rests entirely on the determination of the decomposition of the actinometer solution; this can be done with fairly good accuracy.

It is customary to first calibrate the reference system for the amount of light absorbed per unit time and in experiments thereafter to measure only the irradiation time. Such calibrations have to be made quite frequently however, as the light output decreases on account of the depreciation of the lamp. Under such circumstances it is more desirable to measure the light output during each experiment. The determinations are best performed by irradiating simultaneously the measuring cuvette with the solution and a reference cuvette with actinometer solution of the same optical density and refractive index. The number of quanta absorbed in the respective cuvettes are then proportional, irrespective of fluctuations in light intensity. The constant proportionality factor is determined by irradiating actinometer solutions in both cuvettes. From time to time a check of the value has to be made to correct for possible uneven depreciation of the different parts of the lamp. If, however, the cuvettes are placed near each other such effects will be of small influence. A photocell may be used instead of the reference cuvette (13) and obviously has certain advantages but here again calibrations must be done rather frequently. In this work a reference cuvette has been used.

The cuvettes were thermostated and provisions were made for stirring the solution. The volume of the cuvettes was kept as low as possible, 5–10 ml in order not to use too much material in each irradiation. The cuvettes were either entirely of quartz or provided with quartz windows to transmit the ultraviolet light. A suitable filter was placed between the lamp and the cuvette to remove any possible powerful short wavelength radiation.

The lamps were in the form of spirals, and when high light intensity was required the measuring cuvette could be placed inside the spiral, otherwise it was placed outside. The highest light intensity would be obtained if the lamp could be placed inside the cuvette. However, the large size of the lamp prevented this. Two different experimental set-ups were used.

Apparatus 1

The cuvette was a cylindrical quartz tube, *A*, 10 cm in length and with an inner diameter of 1.09 cm. The walls were 0.8 mm thick. Through a ground joint, *G*, the cuvette fitted into another cylindrical quartz tube, *B*, serving as cooler, with the same thickness of the walls and with an inner diameter of 2.1 cm. The cuvette with the cooler was placed vertically in the center of the lamp (Fig. 7). Two similar cuvettes with coolers could be placed outside the lamp, one or both serving as a reference cuvette. The system was cooled by passing through it a suitable liquid which could also serve as the short wavelength filter. This liquid was placed in a container, surrounded by a water thermostat and circulated through the system by means of an all-glass centrifugal pump. In the circulating system the connections between glass and quartz were simply made by placing the ends of the tubes firmly together inside a short length of rubber or plastic tubing. Such an arrangement provides greater flexibility but it was not found advisable to use strong acetic acid as filter liquid. Acetic acid is otherwise an excellent cut-off filter for wavelengths shorter than 2500 Å (53) and was used in apparatus 2. To get a fairly similar cut-off 50 % sodium acetate in water was used instead (see Fig. 1). No changes in the light absorption of this liquid, due to material dissolved from the tubing, were observed, nor had irradiation any such effect and consequently the same solution could be used for a long time.

While it is advantageous to place the cuvette in the centre of the lamp to get high intensity, a difficulty is encountered in screening the light from the cuvette during

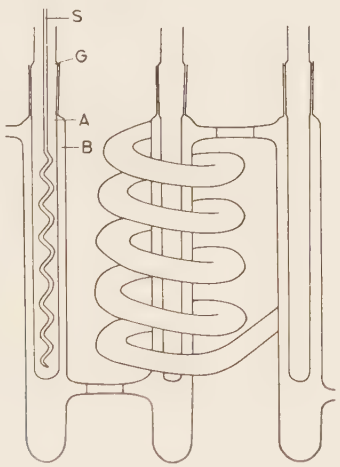


Fig. 7. Apparatus 1.

the warm up period of the lamp. On account of this, at the beginning of an irradiation the solution has to be brought very quickly into the cuvette at the same time as a screen before the reference cuvette is removed. At the end of the irradiation the lamp is simply turned off. After a little training it is possible to get the difference in irradiation time between the two cuvettes well inside one second. If lower intensity is desired one of the outside cuvettes is used for the solution under investigation and the other as before as reference cuvette, no difficulty with screening exists in this case.

The distance between the reference cuvette and the lamp could be varied so as

to give any desired percentage decomposition of the actinometer solution. The ratio of quanta absorbed at the different positions of the cuvette had to be determined.

The stirring was performed through the filling tubes either by small glass stirrers at low speed (about 40 r.p.m.) or by bubbling a slow stream of a given gas, which then also defined the gas atmosphere, through the solution. The danger of getting denaturation effects in protein solutions when too rapid stirring is used has been pointed out by Rideal and Roberts (13). When the glass stirrers were used any desired gas was let in above the surface of the solution. The importance of stirring is evident: Hausser *et al.* (54) have treated the case when completely immobile solutions are irradiated.

The nitrogen used was provided by LKB Laboratory, Stockholm, and contained less than 0.001 % oxygen. Before entering the measuring cuvette it was passed through a wash bottle with sulfuric acid to remove ammonia present and then through a buffer solution identical with that used as solvent for the irradiated solution. The oxygen was of ordinary commercial type and also passed through the proper buffer solution before use.

When the conditions during ultraviolet irradiation are described in the literature one often comes across expressions like "in contact with air", "in an atmosphere of oxygen or nitrogen" etc., without it being stated if the solution is kept saturated with the given gas. It is the author's experience that with a small surface towards the gaseous phase it is not always possible to keep the concentration of the dissolved oxygen constant only by using an oxygen atmosphere or air above the solution even when the stirring is very efficient. To assure such conditions it is necessary to keep a good flow of oxygen or air through the solution. In those cases where the reaction is very sensitive to oxygen it is particularly hazardeous to compare results with those in the literature if the experimental conditions are not the very same.

The lamp was a low-pressure mercury-vapor Hanovia Sc 2537 with instant starting. Buttolph has recently given a detailed description of the general characteristics of such low-pressure lamps (55). The total length was 152 cm, the tube diameter 1.5 cm and the inner diameter of the spiral 7 cm. It was operated through a transformer which also provided the high voltage, 2500 V, required to start the lamp. The lamp is designed to work at room temperature and with normal ventilation. In this investigation the lamp was surrounded by a small lamp house open on two sides and the whole set up was placed in a fume hood to get rid of the ozone formed, care being taken to avoid airdrafts near the lamp.

The time necessary for the lamp to give constant output was determined by irradiating an actinometer solution in the reference cuvette for equal and suitably short times after different warm-up periods. It was found that after a 25 minute warm-up period the output was constant within 5 % and remained so even after many hours of operation. A warm-up period of at least 30 minutes was always used in the experiments.

The manufacturer has determined the energy distribution in the lamp and states that for wavelengths shorter than 3000 Å more than 99.5 % is given off at 2537 Å. At 3132 Å 0.6 % of the energy at 2537 Å is given off and at 3650 Å 0.5 %. The only wavelength shorter than 2537 Å mentioned is 2482 Å where the corresponding value is only 0.01 %. Radiation from the 1849 Å band has been brought to a minimum but is still present as ozone is formed. The total effect of the lamp including visible and infrared is given as 10.7 watt.

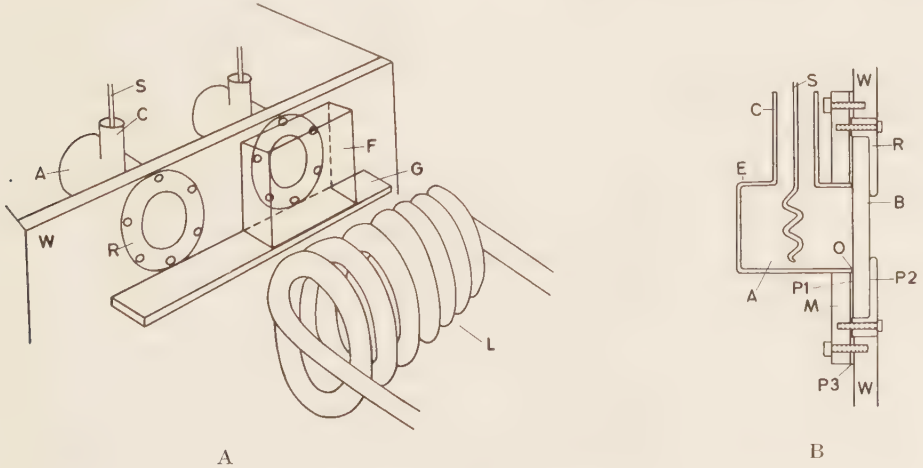
Apparatus 2

Fig. 8 shows the apparatus. The measuring cuvette and the reference cuvette were placed close together on the same side of the lamp which was fixed in a horizontal position. The arrangement is very similar to that of Rideal and Roberts (13) and was also primarily intended for similar experiments.

The two cuvettes were identical, with a length of 3 cm and an inner diameter of 2.2 cm. They were of cylindrical cross-section and had the form of a T-tube one limb, *C*, of which, held in a vertical position, was used for filling purposes. The glass tube, *A*, was cemented into a hole in a metal plate, *M*, in such a way that one end, *O*, protruded slightly in front of the plate. Against this end, which had been ground as plane as possible, a circular quartz window, *B*, was tightly pressed by means of a metal ring, *R*, on which a pressure is maintained by means of six screws threaded into the metal plate. A thin cellophane packing, *P*1 is placed between the quartz and glass surfaces and is protected from the ultraviolet light by the metal ring. The other end, *E*, of the tube had a plane glass window fused onto it. The metal plate holding the cuvette is screwed on to the inside wall, *W*, of the thermostat which contains a hole concentric with the quarts window. *P*2 is a fiber packing and *P*3 a rubber packing.

Normally no difficulties with leakage have been encountered. When monochloroacetic acid is used as actinometer solution, however, it is necessary to change the packing frequently. Between experiments the cuvettes were kept filled with distilled water. For all practical purposes this kind of cuvette is as satisfactory as one entirely of quartz. The thermostating has been adequate, the difference in temperature between the thermostat and the liquid in the cuvette never exceeding 0.1°C. Filters mostly consisting of 21 % acetic acid (53) in a quartz cell could be placed immediately in front of the cuvettes.

In some of the first experiments somewhat thinner cuvettes were used, diameter 3.0 cm, length 1.5 cm. Their cylindrical walls were made of perspex which, however, soon showed signs of cracking from the ultraviolet light.



A B

Fig. 8A and B. Apparatus 2.

The reference cuvette was in a fixed position, screens with suitably large holes were instead placed in front of the cuvette in order to get suitable decomposition. The ratios of the transmittance of the different screens were determined by comparing them two at a time in order of decreasing transmittance. The screen with the lowest transmittance let through about 10 % of the incident light. For very long irradiations the partly decomposed actinometer solution was replaced by a new one at appropriate times instead of using still more opaque screens.

The stirring was performed as in apparatus 1.

The lamp used here was surrounded by a partially open lamp house with a sliding door, and had the same general characteristics as the one in apparatus 1 but was somewhat smaller, length 128 cm and tube width 0.9 cm. The total output was 4.9 watt.

Actinometer solutions

As actinometer solutions both uranyloxalate -oxalic acid and monochloroacetic acid were used, the former in the majority of the irradiations. The light from the lamp is, as previously mentioned, not parallel. It passes, depending on the entrance angle, unequal distances in the solution in the cuvette and therefore it is necessary that both the optical density and the refractive index are very nearly the same for the actinometer solution and the solution to be investigated. For solutions with high optical density, 3–4 at 1 cm, the uranyloxalate–oxalic acid solution is best suited and for those with low optical density the monochloroacetic acid is preferable. The difference in refractive index due to the salt in the buffered solutions was in this connection small enough to be neglected.

Uranyloxalate–oxalic acid

The photochemical decomposition of oxalic acid sensitized by uranylsulfate is described in great detail by Leighton and Forbes (56). The number of quanta absorbed was determined by means of a thermopile using horizontal integration. The concentration was $0.01M$ UO_2SO_4 and $0.05M$ $H_2C_2O_4$ and the decomposed oxalate was determined from the difference in titration value of $0.2N$ $KMnO_4$ between an unirradiated control solution and the irradiated one. The decomposition was small, 5 %. For the quantum yield, ϕ, at 2537 Å and 25°C a value of 0.60 ± 0.03 is given. The dark reaction is negligible and the temperature dependence small. For $\lambda = 3130$ Å it was found to be $\phi_{t+10}/\phi_t = 1.03$.

Forbes and Heidt (57) determined ϕ for a solution which was $0.001M$ in uranyloxalate and $0.005M$ in oxalic acid and found for $\lambda = 2540$ Å at 25 ± 3°C the value 0.65 ± 0.03. They used both titrations with permanganate and electrometric titration with cerium sulfate. Here also the decomposition was around 5 %.

In the present investigation the actinometer solutions were also $0.001M$ in uranyloxalate and $0.005M$ in oxalic acid, the optical density at 2537 Å was 3.60 and the value 0.62 was used for ϕ. This value was also found to agree well when comparisons were made with the quantum yield of monochloroacetic acid, see page 22.

Titrations with $0.01N$ permanganate were used in a number of cases. The amount decomposed was always around 40 % thus making the required amount of permanganate 4–5 ml which could be determined with an accuracy of 1–2 %. A disadvantage is that very frequent standardization of the permanganate is required. Titrations with

0.01*N* ceric ammonium sulfate, which keeps for a long time, were also used. In this case an excess of ceric ammonium sulfate is added and back titration performed with 0.005*N* arsenic oxide in a medium of strong sulfuric acid. As indicators osmium tetra oxide and ferroin (ferrosulfate and *o*-phenantroline monohydrate) were used. The accuracy here is better than 1 %. The obvious advantage is that one titrates directly on the number of moles decomposed and thus can determine even small decompositions accurately. The particulars of this titration method which is used for measuring the light output in flash light experiments by Claesson and Lindqvist will soon be published. Rideal and Roberts used a similar method (13) and recently another variation using light absorption measurements instead of titrations has been described (58).

The necessity of removing the carbon monoxide formed at the decomposition of the oxalic acid before titration has been emphasized (56, 57, 13). Heating for 15 min. at 80°C in the dark is recommended (56). Rideal and Roberts (13) who used large volumes, 50 ml, at irradiation found it necessary to bubble nitrogen through the solution while heating. The present author made a large number of comparative experiments and found that for volumes of 5–10 ml the stirring during the experiment had evidently removed the carbon monoxide as heating and bubbling nitrogen through the solution made no difference. However, in order to be absolutely sure the solutions were always heated for 15 minutes before titration.

Monochloroacetic acid

The photodecomposition of monochloroacetic acid producing chloride ions has been studied by Smith, Leighton and Leighton (59). They used the same technique for evaluating the quantum yield as for the uranylsulfate -oxalic acid actinometer (56). The chloride was determined by potentiometric titration with silver nitrate. This analysis can be performed with very high accuracy. The quantum yield is highly dependent on the temperature; a lower temperature favours deactivation of the excited monochloroacetic acid molecule more than the splitting off of the chlorine. Good thermostating is thus necessary when using this actinometer. The lowest temperature used in the investigation was 25°C, but if the curve showing the temperature dependence of the quantum yield is extrapolated to 21°C one gets a value of 0.285 ± 0.01 for the quantum yield.

The molar extinction coefficient at 2537 Å for monochloroacetic acid is 1.30. An actinometer solution with an optical density of 0.2–0.3 as a reference solution is thus easily obtained. The concentration of the chloride formed is still high enough to permit very accurate determination by means of potentiometric titration with silver nitrate. Monochloroacetic acid solutions of increasing optical density (0.2–3) were irradiated in the cylindrical cuvette placed in the center of the lamp, Fig. 7. A calibration curve for the number of quanta absorbed per ml for any given optical density was obtained. The absorption occurred as if the cuvette had been plane parallel with a thickness 90 % of the diameter of the cylindrical cuvette and with light entering perpendicularily. A detailed account of the experiments which were performed together with Mr. B. Nyman will be given elsewhere. Similar investigations of the effect of non parallel light on the number of quanta absorbed in the plane parallel cuvettes, apparatus 2, showed that for the lower optical densities (< 1) lower values were obtained than would be expected from Beer's law. These results are in accordance with the fact that most of the light has rather low angles of refraction after the passage through the quartz walls.

For optical densities around 3, comparable with those of the uranyloxalate–oxalic acid solution, such a high concentration of the monochloroacetic acid has to be used that the refractive index increases noticeably (60). From the dimensions of the cuvette it is estimated, however, that at these high optical densities the resulting small difference in the angle of the incident light for uranyloxalate–oxalic acid and for monochloroacetic acid is of no importance. Solutions of monochloroacetic acid and uranyloxalate–oxalic acid were irradiated simultaneously at 21°C in apparatus 1 at the same optical density with the monochloroacetic acid in the cuvette in the centre of the lamp. The optical density used was 1.75. The ratio between the quantum yield for uranyloxalate–oxalic acid and that for monochloroacetic acid was found to be 2.18. Using the extrapolated value 0.285 for the quantum yield for monochloroacetic acid the quantum yield for the uranyloxalate–oxalic acid becomes 0.62.

Light scattering corrections

In order to get the correct value of the number of quanta absorbed in a solution which also exhibits light scattering it is necessary to know the amount of scattered light that is reabsorbed. The exact expression for reabsorbed light will depend on the optical density of the solution and on the angle of the incident light; secondary scattering, back-reflection and the distribution of the scattered light will also enter and make the expression rather lengthy. It is, however, possible to make an estimation using some simple reasoning.

For a cylindrical cuvette where the light enters perpendicularly through one of the plane end surfaces we regard a volume element of thickness dl at a distance l from the entrance plane. The intensity is here $I_0 \times 10^{-Ecl}$ (according to Beer's law); E is the extinction coefficient and we have $E = E_a + E_s$, where E_a is the absorption coefficient and E_s the scattering coefficient, I_0 the intensity of the incident light, and c the concentration. The light scattered in the volume element will be $2.303\, E_s I_0 \times 10^{-Ecl} c\, d\, l$, of this a fraction a, ($< \frac{1}{2}$) is scattered back towards the entrance window. We assume an average value of the distance, $lb (b > 1)$, from the volume element to the entrance window. The expression for the amount of light dA which is not reabsorbed will be

$$dA = 2.303\, a\, E_s I_0 \times 10^{-Ecl} c\, d\, l \times 10^{-Eclb}$$

and integrating from 0 to L we get

$$A = \frac{a E_s I_0}{E(1+b)} (1 - 10^{-EcL(1+b)})$$

and the ratio, R, between the light not reabsorbed, A, and the light "absorbed", $I_E = I_0(1 - 10^{-EcL})$, calculated from the extinction coefficient, is easily obtained. For hemocyanin $E_s / E \sim 0.35$, and with $b = 1$ and $a = 0.5$ we get $R = 0.088$ for $EcL = 2$, and $R = 0.157$ for $EcL = 0.1$.

These values for A are considered too high as back reflection and secondary scattering have not been taken into account and also because the value $b = 1$ must be too low and $a = 0.5$ too high. With the high optical densities, $EcL = 2$, used in practically all the experiments in this investigation it was judged that the loss of

light from the cuvettes described would be a few per cent. This correction decreases for hemocyanin as irradiation proceeds, due to the splitting of the molecules. As the correction is relatively small, almost within the experimental errors of the concentration determinations from the ultracentrifuge, and not exactly known, no correction has been applied when calculating the quantum yields. The same applies to serum albumin for the normally used irradiation doses. Oster and McLaren (61) in their investigation of the effect of irradiation on tobacco mosaic virus, where E_s/E was estimated to be 0.5, use a correction factor of about 15 %. The calculation of this value seems, however, not quite clear.

Chapter III

Ultraviolet spectra of irradiated substances containing the protein chromophores

In this chapter we shall consider changes in the absorption of the chromophores of a protein upon irradiation with light of $\lambda = 2537$ Å.

If the changes in the extinction coefficients at the absorbing wavelength (λ 2537 Å) of the different chromophores in a particular protein do not occur in the same ratio the relative amount of energy absorbed in a specific part of a molecule will change as irradiation proceeds. This may cause that the quantum yield for a particular reaction, splitting, aggregation or decrease in enzyme activity varies during irradiation. The question also arises how the changed chromophores are distributed among the molecules, for instance if changed light absorption is combined with change in molecular weight. The following considerations and experiments are made with the purpose to get some knowledge about these matters for *Helix pomatia* hemocyanin and bovine serum albumin especially for the irradiation doses used in chapter VI where the changes in the molecular weight of these proteins are dealt with.

Conclusions have been drawn almost entirely from the observed spectral changes where differentiation between effects due to light scattering and light absorption is possible as the changes in molecular weight also were determined. A detailed analytical investigation of the groups formed upon irradiation has been outside the scope of this work.

Factors determining the spectral changes

As already mentioned, λ 2537 Å has energy enough to break any single bond in the molecule and is absorbed in all the aromatic groups and in the –S–S - bonds and to a small extent in the –CO–NH– bonds and in certain non-aromatic sidechains. It would seem natural that the changes in the spectrum would be due primarily to changes caused by photochemical action in the absorbing groups themselves.

It is, however, well known that energy absorbed in one chromophore may be transferred through the molecule and cause a reaction in some other more sensitive group. The existence of such energy transfer has also been demonstrated in chlorophyll (62), it is of special importance in large molecules. Evans and Gergely (63) have discussed the energy transfer in protein molecules.

In small molecules the possibilities of energy transfer are less but it is known that the energy absorbed in the keto groups of an aldehyde or a ketone does not cause splitting there but in another bond close by (64).

McLaren *et al.* (65, 66) have studied the energy transfer at λ 2537 Å in some aromatic compounds also containing a peptide bond. They irradiated phenylacylalanines as well as acetylalanine and report in all cases the appearance of alanine, indicating a splitting in the peptide bond. The conclusion is drawn that energy transfer from the aromatic group to the peptide bond has occurred, compare also (5). The existence of actual peptide bond absorption at the wavelength used for irradiation is thus not an absolute necessity for the splitting, though of course of importance. The energy transfer in small molecules has also been suggested to occur through actual contact between the groups, being brought about on account of the flexibility of the chain (66, 67).

Though it is true that under identical conditions a given number of chromophores absorb the same number of quanta whether they are present in a large number of small molecules or in a small number of large molecules, the spectral changes may thus be somewhat different on account of the above-mentioned energy transfer.

It is also clear that the constant and more rigid surroundings of the chromophore in a large protein molecule render secondary reactions with the solvent and other molecules more difficult than in the case of a chromophore in a small molecule. The "cage effect", page 40, will make the splitting more difficult in a large molecule. It may be expected that the chromophores at the surface of a molecule and those in the interior will be effected differently. On the whole the study of the behaviour of the chromophores in low molecular weight compounds will give valuable information but never the complete answer to the question of what happens to the same chromophores in large molecules.

The distribution of changed chromophores

The formation of new chromophores as a result of irradiation may occur at the site of the original ones or at a distance therefrom. If the formation of new chromophores is accompanied by a change in the molecular weight, splitting or aggregation, the molecules in the reaction mixture having the original molecular weight will still have their original extinction coefficient. This is true both for large and small molecules.

If on the other hand no change in the molecular weight occurs or if it is independent of changes in the light absorption, matters will be different for molecules with few and many chromophores.

In a solution of irradiated molecules with few chromophores some molecules will have the original extinction coefficient while others will have very different ones. Thus all molecules do not absorb the same number of quanta per unit time and the strongly absorbing molecules will protect the others (inner screening). However, in molecules with a sufficiently large number of chromophores chained together, the primarily changed chromophores are for statistical reasons rather evenly distributed in the molecules and the chances of some molecules absorbing more light than the others will become smaller.

The whole material will register a continuous change in the extinction coefficient. When calculating quantum yields it is of course of utmost importance to be able to decide which of these discussed cases is the relevant one (see chapter IV).

Irradiation experiments

It has long been observed that the ultraviolet spectra of substances containing the aromatic ring or the indol configuration show marked changes upon irradiation (68. 4). The most striking effect is an increase in the minimum around 2500 Å, the maximum is often also enhanced and absorption for wavelengths longer than 3000 Å begins to appear. Similarities between the spectra of irradiated proteins, peptides and amino acids and those of irradiated phenols and quinones have been reported (69). Effects of ultraviolet light and ozone have been compared (70).

In the following the spectral changes of the protein chromophores irradiated when present in small molecules, each containing only one of the chromophores, will first be treated, then the spectra of irradiated *Helix pomatia* hemocyanin and irradiated bovine serum albumin and their corresponding irradiated aromatic amino acid mixtures will be dealt with.

The experimental conditions were as described in chapter II and a temperature of 21°C was used when not otherwise stated. For the proteins the spectra were measured on the same solutions that were investigated in the ultracentrifuge. The buffers used there have been used also for irradiation of the low molecular compounds. The compositions were $0.08M$ acetate + $0.1M$ NaCl at pH 4.4 and 5.3, $0.08M$ phosphate + $0.1M$ NaCl at pH 6.2, and $0.03M$ phosphate–borate + $0.1M$ NaCl at pH 8.2. The buffers showed negligible absorption at 2537 Å, both unirradiated and irradiated.

Detailed information of the proteins used are found in chapter VI. The low molecular compounds were of good commercial quality (Merck and Hoffman La Roche) and used without further purification.

When the effect of irradiation has to be correlated with the number of quanta absorbed, the author has in most cases chosen to express the latter in terms of quanta absorbed per ml solution per optical density unit of the unirradiated solution at 2537 Å. The notation Q/d_{2537} can be regarded as a measure of the number of quanta absorbed per chromophore originally present in the solution, and can when the molar weight is known easily be transformed to for instance Q/N^0 where N^0 is the number of molecules present at the start. It seemed practical to use Q/d_{2537} as comparisons are made both between substances of very different molecular weight and substances with different extinction coefficients. In the text the notation Q/d is used for Q/d_{2537}. The intensity will be denoted by dQ/dt and is expressed as quanta absorbed per ml and minute at total absorption.

Tyrosine

Both tyrosine and glycyltyrosine were studied. Photo-oxidation was judged to be less important both for glycyltyrosine and for tyrosine combined in a protein than for tyrosine itself, as glycyltyrosine is much more slowly oxidized by tyrosinase than tyrosine itself where the free amino group plays an important role in the oxidizing scheme (31). Glycyltyrosine is also much more soluble in the buffers used. Irradiations were performed at pH 4.4, 5.3, 6.2, and 8.2 and in an atmosphere of air, oxygen and nitrogen. The spectra in the region 2300–4000 Å were determined and the extinction coefficient E at 2450 (min), 2537, 2760 (max) and 3100 Å were plotted against Q/d. These results are seen in Figs. 9 and 10.

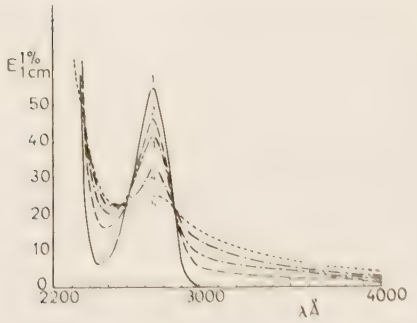


Fig. 9. Absorption spectra of glycyltyrosine irradiated at pH 6.2 in oxygen saturated solution. $Q\ d_{2537} = 0$, 9, 26, 57 and $136 \times 10^{18}\ h\nu$/ml for curves 1 to 5 resp.

A decrease of the maximum, an increase of the minimum which was shifted towards longer wavelengths and an absorption appearing for $\lambda = 3000$ Å was observed. The increase at 2450, 2537 and 3100 Å is somewhat larger for higher pH's and in an atmosphere containing oxygen. The decrease at 2760 Å is correspondingly smaller. It is noticeable that the effect of oxygen becomes appreciable only in alkaline solution. The increase at 2450 Å and below 3000 Å when irradiated in nitrogen can hardly be the effect of traces of oxygen. The enormously increased oxygen concentration when oxygen is bubbled through the solution should in that case also produce a very much greater effect.

The solutions were slightly yellow after irradiation, no smell was observed. No significant changes were observed in the spectrum when the solutions were allowed to stand for a few days. Most of the points correspond to a concentration of 0.02 % at irradiation and an intensity of $5.2 \times 10^{18}\ h\nu$/ml min but a few with higher concentration 0.25 % also fall reasonably well on the curve as well as some irradiated at the lower intensity $0.55 \times 10^{18}\ h\nu$/ml min. The curves indicate a one-hit process (see p. 42). However, as the optical density at 2537 Å is not constant, deviation from the theoretical curve must occur. The extinction coefficient used for a 0.1 % glycyltyrosine solution is 1.125 at 2537 Å. The quantum yield, ϕ, for the decrease of the maximum $\lambda = 2760$ Å measured at the beginning of the irradiation is found to be 0.038.

As the sole purpose of this investigation was the study of the spectral changes, a complete analysis of the products resulting from the irradiation was not undertaken. It may, however, be mentioned that when the irradiated solution was examined with paper chromatography no traces of either glycine or tyrosine could be detected. The same has been noted by McLaren *et al.* (66). Carpenter on the other hand (71), when irradiating glycyltyrosine in monolayers, reported the appearance of glycine and tyrosine. In the present investigation a solution irradiated to a point where further changes in the spectrum were negligible (see Fig. 9) had given off about 50 % of the nitrogen as ammonia, determined according to Conway. No spots were detected by means of ninhydrine in the paper chromatogram, showing that very little amino nitrogen could be present. Frontal analysis on active carbon on which aromatic groups have a very strong adsorption showed that more than 90 % of the material, which contained several components, had a much lower adsorption than that of glycyltyrosine itself and comparable to that of aliphatic peptides or amino acids. The light absorption at 2450–4000 Å was very small for this fraction. The rest, 10 %, was more strongly adsorbed on carbon than glycyltyrosine and was responsible for most of the absorption in the spectrum including that at 2450 and below 3000 Å.

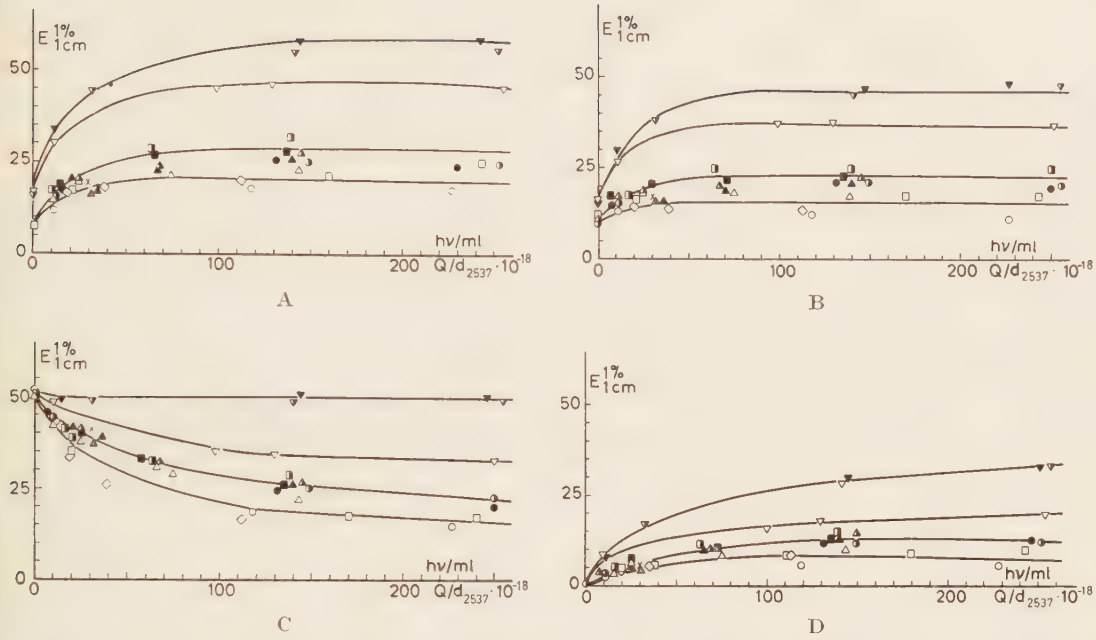


Fig. 10. $E^{1\,\%}_{1\,cm}$ as a function of Q/d_{2537} for irradiated glycyltyrosine, ○ at pH 4.4, △ at pH 5.3, □ at pH 6.2 and ▽ at pH 8.2, all in nitrogen atmosphere. The corresponding half-filled symbols refer to the same pH's but in oxygen or air atmosphere. The filled symbols refer to irradiations when the solution is bubbled through by oxygen, × different intensity, ◬ and ◇ different concentrations. Wavelength **A** 2450 Å, **B** 2537 Å, **C** 2760 Å and **D** 3100 Å.

The aromatic character seems to have disappeared completely. Whether the molecule splits at the peptide bond or in another bond cannot be decided until the photochemical reaction has been completely explained. According to Weizmann *et al.* (72) nitrogen would be more readily split off from the amino group than from the peptide bond. The amount of ammonia found was proportional to the decrease of the maximum suggesting that the two reactions are going parallel. The infrared spectrum was taken in one case on the changed material, isolated by means of frontal analysis on carbon. Products of simple structure were indicated.

Tyrosine in nitrogen atmosphere shows the same general change of the absorption spectrum as glycyltyrosine: increase in the minimum, decrease in the maximum, though not as pronounced as for glycyltyrosine, and increased absorption at $\lambda > 3000$ Å (see Fig. 11). The concentration used was 0.01 % and the extinction coefficient at 2537 Å was 0.181. When oxygen is present the maximum is in addition enhanced and the other effects increase due to the greater tendency of these compounds to be oxidized. More or less similar effects have been observed earlier (69). Dioxyphenylalanines have been identified in solutions irradiated in air (73). When irradiated in pure water glycyltyrosine and tyrosine show somewhat larger effects for the same amount of absorbed quanta than when buffer solutions at the same pH are used. Incidentally, irradiated phenol shows very nearly the same spectral changes as irradiated tyrosine.

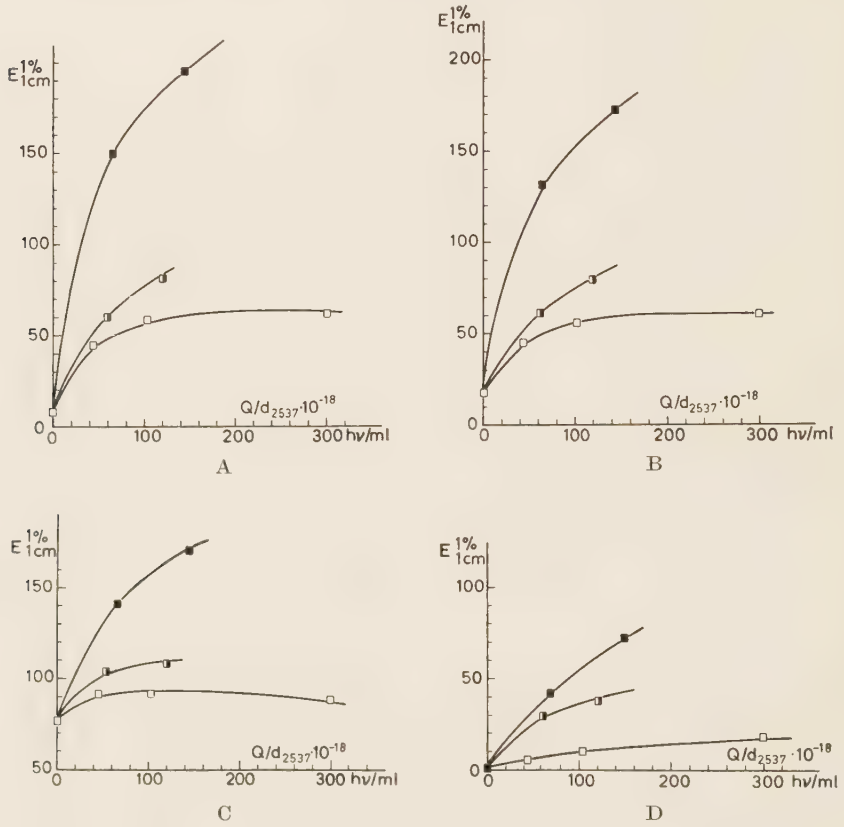


Fig. 11. $E^{1\%}_{1\,cm}$ as a function of Q/d_{2537} for tyrosine. Legend as in Fig. 10.

Phenylalanine

Phenylalanine is known (74) to be highly resistant to oxidizing agents and was studied in its uncombined form. The irradiations were performed at pH 6.2 and 8.2 in air, oxygen and nitrogen at a concentration of 0.3 %. Fig. 12 shows a spectrum and Fig. 14 gives the variations of E (1 %, 1 cm) with Q/d at the wavelengths 2350 (min.), 2537, 2800 and 3100 Å. The extinction coefficient at 2537 Å was 0.80 for a 0.1 % solution. The minimum was shifted towards longer wavelengths. A large increase over the whole spectrum and an additional absorption for $\lambda > 3000$ Å is observed both in nitrogen and in oxygen where the effect is somewhat larger. In air the same kind of behaviour has been observed by McLean and Giese (69). Fig. 13 shows the enormous increase in E for a 0.025 % solution for which $Q/d \sim 294 \times 10^{18}$ *hν* ml. Around 3100 Å a shoulder is apparent, which, however, gradually flattens out when the solution is allowed to stand. After about 24 hours, see Fig. 12, no further change is observed. A slight increase at wavelengths lower than 2800 Å occurs at the same time. Irradiated phenylalanine solutions have a striking smell and a yellow colour. The general effects of irradiation are somewhat larger in pure water. McLaren *et al.* (65) have investigated phenylalanine in combined form as

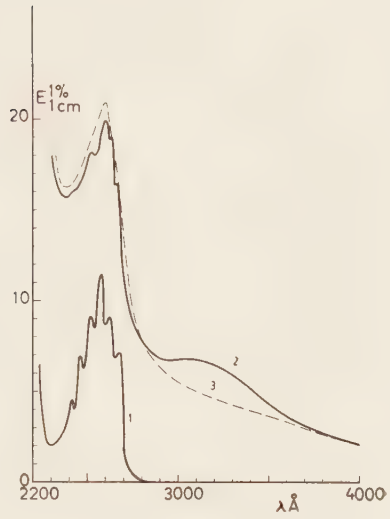


Fig. 12. Absorption spectra of **1** unirradiated phenylalanine pH 6.2, **2** irradiated phenylalanine pH 6.2 bubbled through by oxygen, $Q\ d_{2537} = 49 \times 10^{18}\ h\nu$/ml measured immediately after irradiation, **3** same as **2** but measured 24 hours later.

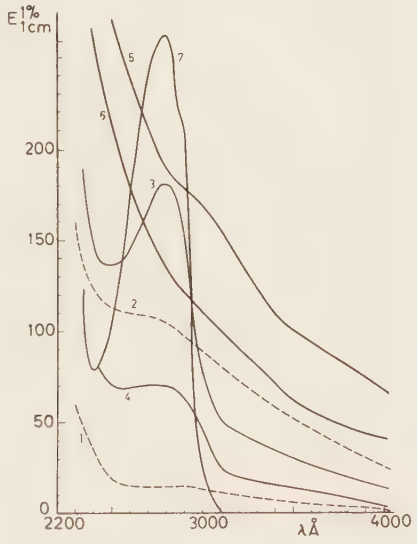


Fig. 13. Absorption spectra of irradiated **1** glycyltyrosine, **2** phenylalanine, **3** tryptophan in nitrogen atmosphere unfiltered, **4** same as **3** filtered, **5** tryptophan in oxygen atmosphere unfiltered, **6** same as **5** filtered, **7** unirradiated tryptophan. $Q/d_{2537} = 240$, 294, 79, 79, 77 and $77 \times 10^{18}\ h\nu$/ml for curves **1** to **6** resp.

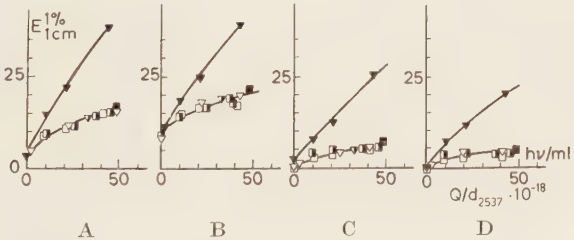


Fig. 14. $E^{1\%}_{1\,cm}$ as a function of Q/d_{2537} for phenylalanine. Wavelength **A** 2350 Å, **B** 2537 Å, **C** 2800 Å and **D** 3100 Å, rest of legend as in Fig. 10.

acetylated phenylalanine and the spectral changes observed seem to be rather similar to the above mentioned. Water was used as solvent and the solutions remained uncoloured.

Frontal analysis on active carbon of the irradiated phenylalanine solution showed 3 fractions. The first was present in low concentration and exhibited low adsorption on the carbon and low light absorption. The second fraction consisted of unchanged phenylalanine. The third fraction which was partly responsible for the strong light absorption only amounted to about 5 % of the total concentration even for Q/d around 20×10^{18} $h\nu$/ml and showed considerably higher adsorption on the carbon than the phenylalanine itself. 4–5 % of the nitrogen had been given off as ammonia at the last-mentioned Q/d value.

As to whether the products with very high light absorption have any similarity to those formed when benzene is irradiated in organic solvents (75) awaits further investigation.

Tryptophan

Tryptophan was also irradiated at pH 6.2 and 8.2 in oxygen and nitrogen at a concentration of 0.02 %. The extinction coefficient at 2537 Å was 1.48 for 0.01 % solution. An increase in the minimum, a decrease at the maximum and absorption appearing at $\lambda > 3000$ Å is found also here. The decomposition of tryptophan is evidenced by the disappearance of the absorption band at 2800 Å, see Figs. 13 and 15. An aggregation reaction proceeds at the same time, and finally precipitation occurs. In nitrogen this aggregation is more marked than in oxygen and already at $Q/d = 20 \times 10^{18}$ $h\nu$/ml the solution shows opalescence (yellow white). In oxygen, formation of small amounts of a brown yellow precipitate occurs at about $Q/d = 50 \times 10^{18}$ $h\nu$/ml. In Fig. 13 the spectra of two tryptophan solutions unfiltered and filtered irradiated in nitrogen and oxygen at pH 6.2 and with $Q/d \sim 75 \times 10^{18}$ $h\nu$/ml are seen. The aggregates in nitrogen evidently have a less flattened out spectrum than the unaggregated material where the absorption band is hardly noticeable. In oxygen where aggregation sets in later the chromophores have already changed considerably before they form part in the aggregates and thus the difference between

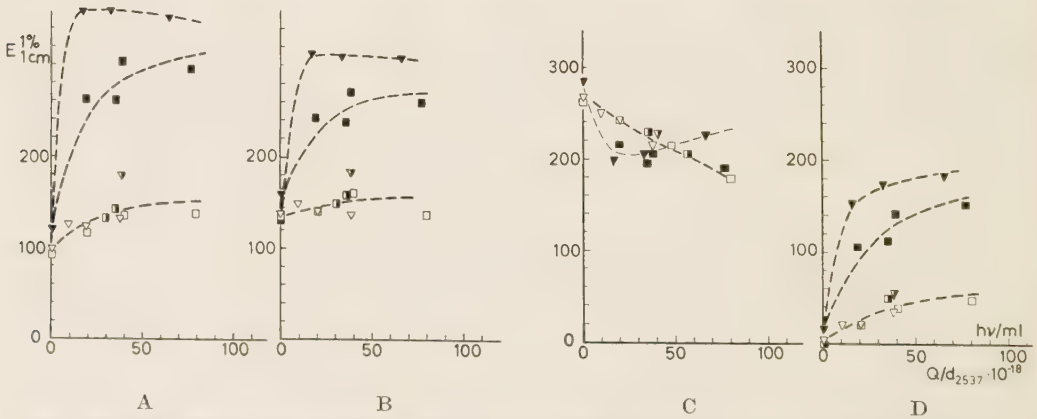


Fig. 15. $E^{1\%}_{1\,cm}$ as a function of Q/d_{2537} for tryptophan. Wavelength **A** 2450 Å, **B** 2537 Å, **C** 2800 Å and **D** 3100 Å, rest of legend as in Fig. 10.

filtered and unfiltered material is not as striking. The same general trend in the absorption changes as reported above is also found when a very dilute solution, 0.001 %, is irradiated, precipitation is here negligible. The results are in agreement with those of Mishchenko (76) and McLean and Giese (69).

The –S–S– bond

The spectral changes due to the action of λ 2537 Å on the –S–S– bond have not been studied by the present author. That the –S–S– bond in cystine is broken upon irradiation with ultraviolet light has, however, been demonstrated for instance by Schochen (77) who found cysteine after irradiation. The –S–S– bond in cyclic structures is likewise broken by irradiation at 2537 Å (78) shown by the disappearance of the characteristic absorption at 2500 Å. The fission of –S–S– bonds in a protein would consequently cause decrease of the minimum in the protein spectrum.

The peptide-bond

The changes occurring when the peptide bond itself is irradiated was studied for leucylglycine and clupein and were found to be practically negligible compared to those mentioned earlier. Irradiation of leucylglycine produces, after the absorption of a very great number of quanta, the effects shown in Fig. 3. Irradiated clupein also shows a general increase in the spectrum, see Fig. 3. Irradiated leucylglycine has previously been reported not to show any change at all (5). However, the number of quanta absorbed were probably very low compared with those in the above-mentioned experiments.

When investigated with paper chromatography only slight traces of leucine and glycine were detected. Ammonia was given off and products containing amino nitrogen but moving faster than leucylglycine on the chromatogram (butanol–acetic acid used as solvent) were found. On active carbon these products had a higher adsorption than leucylglycine.

If the action of light on leucylglycine is the same as that on phenylacylalanines, where alanine was found, page 24, glycine and leucine ought to show up. As only traces have been found it is difficult to decide if a splitting at the peptide bond occurs to any great extent. As previously mentioned neither glycine nor tyrosine is detected in the irradiation mixture of glycyltyrosine. The evidence for splitting in the peptide bond for irradiated peptides is mainly indirect and proof of a more direct character is desirable.

Concluding remarks

It follows from the foregoing that the changes observed in the spectra are not primarily caused by oxygen though the presence of the latter increases the effect. For irradiations performed with the whole ultraviolet spectrum the same has been observed for instance by Magill *et al.* (68) and by Mishchenko (76). The amounts of ozone formed during irradiation is too small to have any influence on the reaction (70).

There exists, however, a certain similarity between the effects of ultraviolet light and those of ionizing irradiation; for example the strong increase in the minimum at about 2500 Å and the more or less pronounced disappearance of the maximum at 2800 Å (79).

For ionizing irradiation the changes are caused indirectly by OH radicals formed from the water and when oxygen is present also by O_2H radicals and H_2O_2 molecules.

As to whether secondary reactions with radicals play an important role for the change in the ultraviolet spectrum at the irradiation with ultraviolet light is still an open question.

The presence of oxygen in the solvent ought to have less influence on the changes in the chromophores of a large molecule than of a small one. Consequently the previous results in nitrogen seem to be more relevant than those in oxygen for comparison with proteins. It has been found that for Q/d values up to 10 which are those most frequently used when studying the changes in molecular weight, chapter VI, the changes in light absorption at λ 2537 Å of the tryptophyl groups are rather small, those of the tyrosyl groups are somewhat larger whereas those of the phenylalanyl groups are comparatively large. The light absorption of other groups is considered negligible compared to those mentioned above.

For a protein containing the above-mentioned amino acids one would thus expect that if the effect on the incorporated acids would be approximately the same as that demonstrated above there would be an increase at the minimum at 2450 Å (the decrease due to the fission of –S–S– bond is generally not large enough to be of importance in this connection), additional absorption at $\lambda > 3000$ Å and a decrease or increase at 2800 Å, depending on which acids are the predominant ones. A flattened-out spectrum will be the final result. Phenylalanine residues might give rise to particularly high extinction coefficients.

Helix pomatia hemocyanin

The spectra of irradiated hemocyanin were studied after irradiation in air at pH 5.3, 6.2 and 8.2 in the above mentioned buffer systems (p. 25) and also in distilled water. Irradiation in oxygen and nitrogen showed that the atmosphere has little influence on the spectrum as long as aggregation does not occur. The aromatic amino acid mixture used for comparative irradiation was made up according to Table 2. The extinction values, $E^{1\,\%}_{1\,\mathrm{cm}}$, refer in this case to concentrations of the amino acids corresponding to those in a 1 % protein solution.

The tryptophyl groups are here the main absorbers and consequently hemocyanin ought to show a rather small change at λ 2537 Å upon irradiation. For the amino acid mixture this is also the case but for the protein itself the change is larger.

The particulars of the changes in the molecular weight properties upon irradiation are given in chapter VI. Only those facts necessary for understanding the spectral changes will be mentioned here. At pH 5.3, 6.2 and in distilled water the molecule normally has a molecular weight of 9×10^6. Irradiation at pH 6.2 produces a splitting into subunits of half this molecular weight whereas the splitting is less marked at pH 5.3 and in distilled water. Aggregation will also take place especially at pH 5.3 but is not important for the lower Q/d values at the high salt concentrations used here. At pH 8.2 where the molecular weight normally is about 1×10^6 splitting into a lower component (with approximately half the molecular weight) occurs. These facts have to be kept in mind when comparing plots of the extinction coefficient E against Q/d for the irradiated hemocyanin and corresponding irradiated aromatic amino acid mixture, see Figs. 16 and 17. The concentrations at irradiation were around 0.2 % and no correction for scattered light was applied, chapter II, page 23.

The general changes in the spectrum of hemocyanin upon irradiation are best studied when the irradiation is performed in distilled water in cases where there are no great

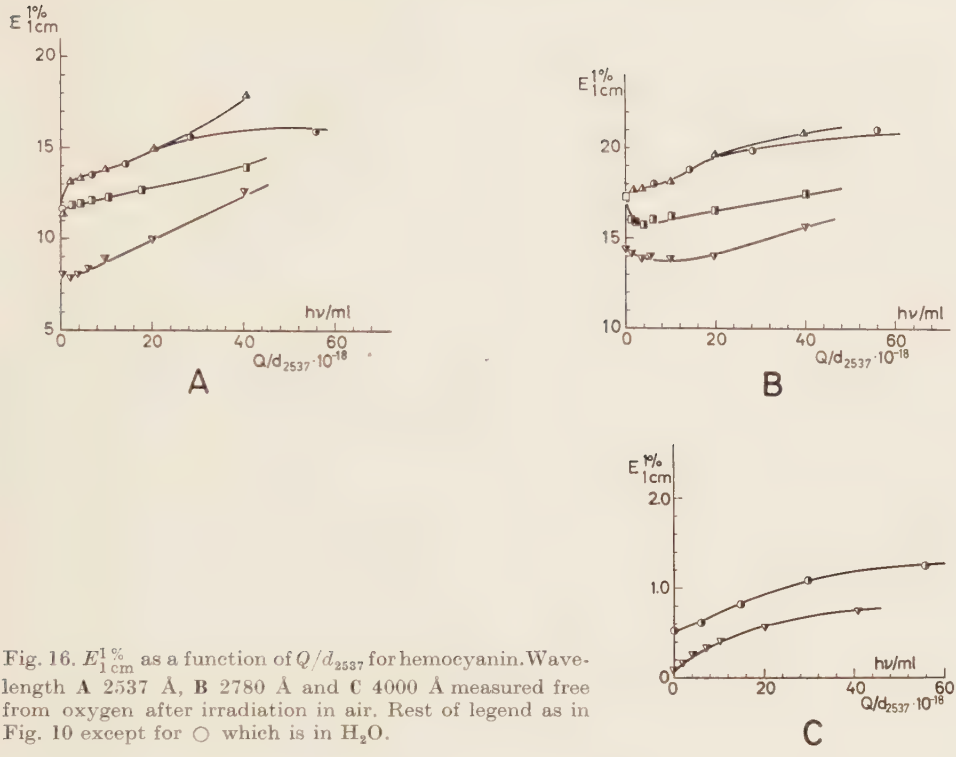


Fig. 16. $E^{1\%}_{1\,cm}$ as a function of Q/d_{2537} for hemocyanin. Wavelength **A** 2537 Å, **B** 2780 Å and **C** 4000 Å measured free from oxygen after irradiation in air. Rest of legend as in Fig. 10 except for ○ which is in H_2O.

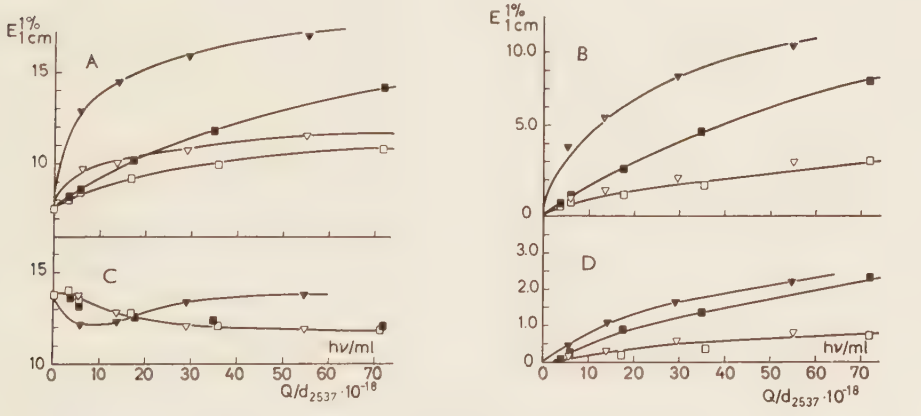


Fig. 17. $E^{1\%}_{1\,cm}$ as a function of Q/d_{2537} for the aromatic amino acid mixture corresponding to hemocyanin. Wavelength **A** 2537 Å, **B** 3100 Å, **C** 2750 Å and **D** 4000 Å, rest of legend as in Fig. 10.

changes in the molecular weight. A general increase of the extinction coefficient over the whole spectrum is observed, the increase at 2537 Å is larger than that at 2780 Å. This results in a spectrum where the distinct maximum has disappeared. Comparisons with the corresponding amino acid mixture show that the changes are larger for the protein. Above 3000 Å the spectrum of the reduced form of hemocyanin gives better information of changes in the protein spectrum itself than the normal oxidized form where the copper band absorption decreases upon irradiation thus making comparisons difficult (see chapter VI). As mentioned before, page 14, in order to get the reduced form of the irradiated hemocyanin, nitrogen is slowly bubbled through the solution. However, during this operation denaturation occurs more easily for irradiated than for unirradiated protein and great care has to be exercised. The extinction coefficient at 4000 Å for the reduced form is equal to the scattering coefficient plus any small absorption produced by irradiation. The measured values (Fig. 16) do not indicate increased scattering, the small increase is of the same order as that of the amino acid mixture. The ultracentrifuge diagrams do not show aggregation either and thus the conclusion is that the somewhat larger increase in the protein spectrum is not caused by light scattering. At pH 5.3 which is close to the isoelectric point 5.05 (12) the changes are first rather similar to those in distilled water but when aggregation starts, evidenced both at wavelengths above 4000 Å and in the ultracentrifuge diagrams, the extinction coefficient of course increases more.

At pH 6.2 a decrease at 2780 Å, due to the lower light scattering of the subunits, is noticed for low $Q\ d$ values. At higher $Q\ d$ values the extinction coefficient increases. However, for very high $Q\ d$ values it again decreases somewhat. At 2537 Å the increase of the absorption coefficient is already at the beginning larger than the decrease of the scattering coefficient and thus a continuous increase is noticed at this wavelength. The final spectrum for very high Q/d values has a flattened out appearance, see Fig. 18.

At pH 8.2 the decrease at 2780 Å at low Q/d values is very similar to that for tryptophan and the amino acid mixture. Increased absorption over the whole spectrum will also appear in this case for high Q/d values.

Low molecular weight components cannot be responsible for the spectral changes as dialysis does not bring about a change in the spectrum.

The irradiated solutions have no special smell, but a slight yellow colour may be detected for higher Q/d values.

No time-dependent changes occur in the spectra of irradiated hemocyanin during the first 24 hours after irradiation in the cases where aggregation has not been observed. However, if during irradiation aggregation starts, it seems to proceed at a slow rate producing a slight increase in the extinction. After long standing (several months) smaller or larger amounts of precipitates are found in all irradiated solutions, which shows that a tendency towards aggregation always is present in irradiated solutions.

It is seen from Table 2 that at a $Q\ d$ value of $7.0 \times 10^{18}\ hr$ ml which is of the usual order of magnitude employed at the experiments in chapter VI, very small changes in the amino acid composition have occurred in the amino acids determined there. Tryptophan may of course be more destroyed.

As expected, aggregation occurs more readily at pH's close to the isoelectric point and at low salt concentrations. The conditions for aggregation have not been investigated in detail; it has however been noticed that aggregation is more pronounced in nitrogen than in oxygen. When the filterable aggregates are removed the same

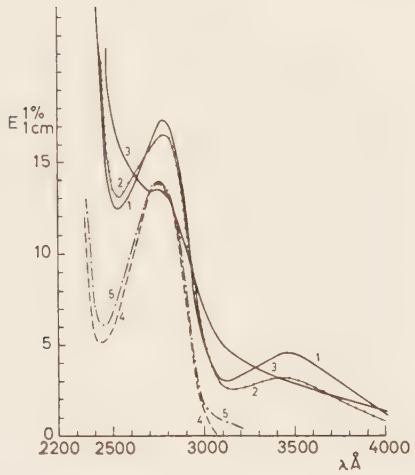


Fig. 18. Absorption spectra at pH 6.2 of hemocyanin. **1** Unirradiated, **2** and **3** irradiated $Q/d_{2537} = 9.3$ and 125×10^{18} $h\nu$/ml respectively of the corresponding aromatic amino acid mixture, **4** unirradiated, **5** $Q/d_{2537} = 9.7 \times 10^{18}$ $h\nu$/ml.

phenomena as noticed for tryptophan is observed, see Fig. 13. The spectrum of the solutions irradiated in nitrogen atmosphere has, before filtration, a distinct maximum left at 2780 Å but for the filtered solution this has completely disappeared, showing that the aggregates have a less flattened out spectrum than the molecules of lower molecular weight in the filtrate. For the spectrum of solutions irradiated in oxygen with approximately the same Q d values the maximum is less marked already in the unfiltered solution and a rather small change is noticed after filtration. The spectrum of the filtered solution shows, however, a more flattened out appearance than that of the unfiltered solution. It seems thus likely that a certain amount of protection against irradiation effects is offered in the very large aggregates.

It is seen from Fig. 18 that the copper band at 3460 Å decreases upon irradiation, compare also page 81.

The question whether the changes in absorption are connected with the split products or the aggregation products is of utmost importance for the later studies

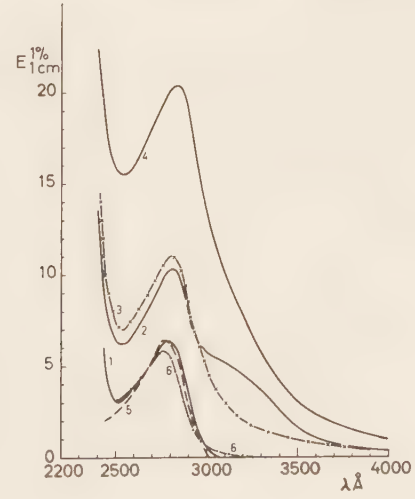


Fig. 19. Absorption spectra at pH 6.2 of bovine serum albumin: **1** unirradiated, **2** $Q/d_{2537} = 7.1 \times 10^{18}$ $h\nu$/ml measured immediately after irradiation, **3** same as **2** but measured 24 hours later, **4** $Q/d_{2537} = 133 \times 10^{18}$ $h\nu$/ml; and of the corresponding amino acid mixture: **5** unirradiated, **6** $Q/d_{2537} = 7.0 \times 10^{18}$ $h\nu$/ml. Air atmosphere except in **4** where oxygen was bubbled through.

in chapter VI. As the absorption changes are found also for hemocyanin in water when the molecular weight remains constant it seems unlikely that they result from splitting or aggregation. Comparisons of the absorption coefficient for different fractions of the irradiated material at pH 6.2 are difficult because of the effects of light scattering. However, a hemocyanin solution irradiated at pH 8.2 where the light scattering is very much smaller, was fractionated by means of frontal analysis on active carbon. The first fraction, with the lowest adsorption, contained proportionally a smaller amount of the component with lower molecular weight (the split product) than later fractions and the original solution. This was shown by the ultracentrifugation diagrams. However, within the experimental errors this first fraction had the same spectrum as the later fractions and the original solution, see Table 3.

The above-mentioned facts suggest that an even change in the light absorption occurs for the whole material and that except for the very large aggregates we have at least statistically the same absorption spectrum for all the molecules in the mixture. The fact that similar conclusions can be drawn for irradiated serum albumin, also makes the assumption for hemocyanin more justified.

Bovine serum albumin

Bovine serum albumin was irradiated in a number of different media. The absorption will be predominantly that of the tyrosyl and phenylalanyl groups, see Table 2, and thus a proportionally larger amount of energy ought to be absorbed at the sites

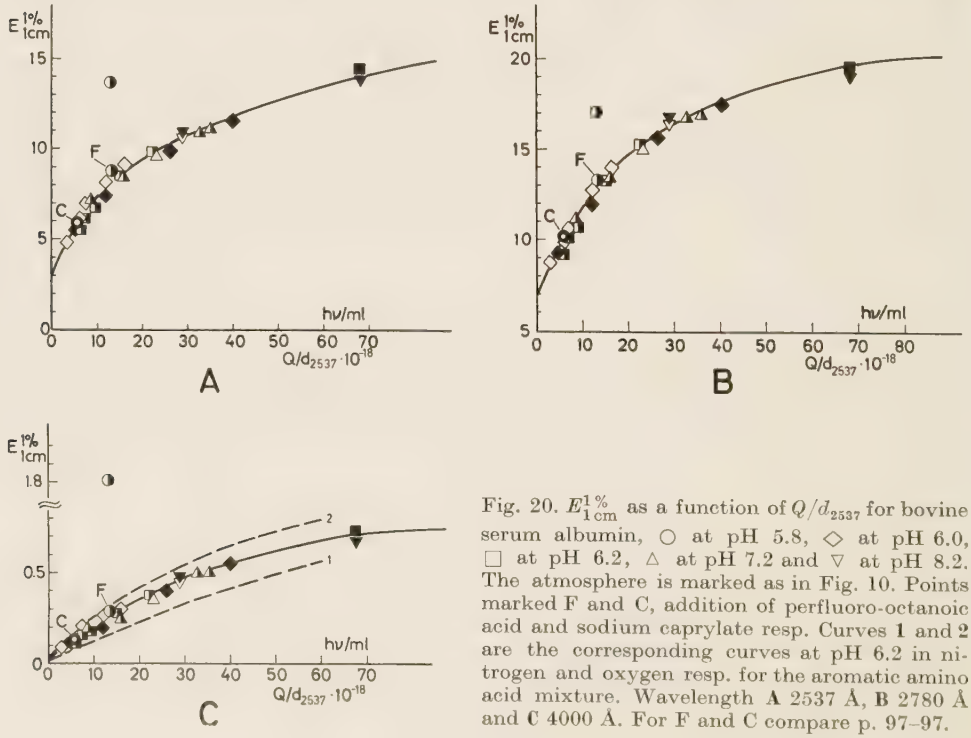


Fig. 20. $E^{1\%}_{1\,cm}$ as a function of Q/d_{2537} for bovine serum albumin, ○ at pH 5.8, ◇ at pH 6.0, □ at pH 6.2, △ at pH 7.2 and ▽ at pH 8.2. The atmosphere is marked as in Fig. 10. Points marked F and C, addition of perfluoro-octanoic acid and sodium caprylate resp. Curves **1** and **2** are the corresponding curves at pH 6.2 in nitrogen and oxygen resp. for the aromatic amino acid mixture. Wavelength **A** 2537 Å, **B** 2780 Å and **C** 4000 Å. For F and C compare p. 97–97.

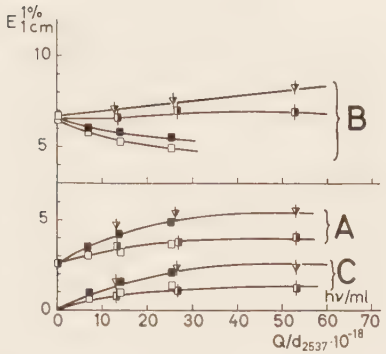


Fig. 21. $E^{1\,\%}_{1\,\mathrm{cm}}$ as a function of Q/d_{2537} for the aromatic amino acid mixture corresponding to serum albumin. Wavelength **A** 2537 Å, **B** 2780 Å and **C** 3100 Å. Rest of legend as in Fig. 10 except that points with a line mark amino acid mixture with tyrosine, the other with glycyltyrosine.

of the phenylalanyl groups as irradiation proceeds. The spectrum of irradiated bovine serum albumin shows very much larger changes than that of the corresponding aromatic amino acid mixture, see Figs. 19, 20 and 21. For lower Q/d values the atmosphere has little influence on the spectrum. As aggregation is the result of irradiation (see chapter VI) light scattering will have influence on the spectrum at sufficiently high Q/d values.

The concentration at irradiation was 0.2–1 % and the pH's used were predominantly 6.2 and 6.0, the latter buffer contained 0.1*M* phosphate and 0.3*M* KCl. Two different amino acid mixtures were used, one containing free tyrosine and the other glycyl tyrosine. The composition was chosen according to Table 2 (see also page 32 for the meaning of $E^{1\,\%}_{1\,\mathrm{cm}}$ in this case). As the two irradiated amino acid mixtures showed rather similar changes, the effect for serum albumin cannot be due to the presence of free tyrosine.

A large increase over the whole spectrum from 3500 Å downwards is the general effect. The change in the minimum is proportionally larger and the result is a more and more flattened out spectrum. Immediately after irradiation a shoulder at about 3100 Å is observed; it flattens out during the next 24 hours (the solution is kept in a refrigerator at 4°C) and a noticeable increase of the extinction coefficient for wavelengths at and below 2800 Å occurs at the same time, see Fig. 19. The situation is very reminiscent of that for irradiated phenylalanine and it would be tempting to assign the change to the effect of irradiation on the phenylalanine in the protein, possibly enhanced by energy transfer ((2), p. 139).

It is interesting to note that a shoulder at 3250 Å has been observed by Haas, Sizer and Loofbourow (31) for tyrosyl groups, oxidized by tyrosinase, and has been found to be especially pronounced for tyrosyl residues with free amino groups.

Light scattering cannot be the cause of the large effect at low Q/d values as the extinction coefficient at 4000 Å is quite low. Furthermore, since dialysis does no change the spectrum, low molecular compounds cannot be responsible either.

The changes observed may also be attributed to some new chromophores in the protein for the formation of which the special way in which the chromophores of the amino acids are arranged in the protein is essential. Hemocyanin showed for instance a much smaller change than serum albumin. The energy transfer to other amino acids which normally do not absorb at 2537 Å is always a possibility and especially the role of histidine ought to be investigated in this connection. It is known that this amino acid upon irradiation with the whole ultraviolet spectrum

shows appreciable absorption around 3100 Å (36). It may not only be a coincidence that pepsin, which has a low histidine content (0.9 g/100 g protein) (80) compared to bovine serum albumin (3.8 g/100 g protein), does not show the very much increased absorption at 2780 Å (81, 82). In the minimum the absorption is as usual increased. For hemocyanin a disappearance of the strong tryptophan absorption may counteract the effects observed for serum albumin which has a low tryptophan content. Nor does chymotrypsin, which has a high tryptophan content ~ 6 g/100 g protein and a comparatively low histidine content ~ 1.26 g/100 g protein (80), exhibit any strong absorption increase at 2780 Å (83).

The changes may also be caused by the presence of small amounts of foreign material. It has for instance been reported (84) that carbohydrate solutions after exposure to ultraviolet radiation show appreciable absorption in the ultraviolet. As such compounds have next to no absorption in their unirradiated form the amount of light absorbed by them in a solution containing the very highly absorbing aromatic groups must be small and large irradiation effects caused by them are therefore difficult to explain except by energy transfer. The role that metal impurities may play is still unknown.

Opalescence and finally precipitation is observed in the irradiated solutions for high Q/d values. The occurrence of this is highly dependent upon the pH; near the isoelectric point comparatively small Q/d values are needed. The high extinction coefficient at pH 5.8 in Fig. 20 demonstrates this.

It is also noticed for serum albumin that whereas the changes in the spectra at lower Q/d are the same in an atmosphere of nitrogen, oxygen or air, association to large aggregates is favoured in nitrogen. Upon filtration the same phenomena as described for tryptophan and hemocyanin is found.

It should be pointed out that Becker and Szendrö (86) when irradiating egg white and McLaren *et al.* (83) when irradiating chymotrypsin found that the maximum around 2800 Å persisted when the irradiation was performed in nitrogen but that it disappeared in oxygen.

In order to investigate the extinction coefficient of molecules of different molecular weight in an irradiated serum albumin solution the latter was run for 2 hours in a Spinco preparative centrifuge at 35,000 r.p.m. The top and bottom fractions were pipetted off and run in the ultracentrifuge. The spectra of the fractions were determined and it was found that irrespective of the molecular weight distribution the spectrum of solutions of the same total concentration was the same. This suggests that the spectral changes have occured rather evenly for the whole material. Table 3.

Sanigar *et al.* (10) after irradiating human serum albumin at pH 7.4 found effects similar to those described above. The large increase over the whole spectrum was observed. The decreasing absorption at about 3100 Å is, however, reported only for solutions irradiated at 0°C. The same authors also made irradiations at pH 5.4 and got a larger increase than at 7.4. It seems more probable that this increased effect is due to light scattering at the lower pH than to differences in the absorption spectrum at the two pH's. The authors themselves interpret the result as showing that the action of light is greater at 5.4 than at 7.4, however, they do not consider the effect of light scattering.

Duggan, Giese and Luck (85) also discuss possible reasons for the spectral changes of irradiated serum albumin. They found that the increase in the extinction of an irradiated serum albumin solution became smaller in a medium containing sodium caprylate which was thus supposed to have some protective effect. However, the

Table 3. The extinction coefficient E (1 %, 1 cm) at certain wavelengths for irradiated *Helix pomatia* hemocyanin and bovine serum albumin containing different proportions of "unchanged" material.

Substance	Fraction	"Unchanged" material in %	E	
			2537 Å	2780 Å
Hemocyanin component III (eighths) irradiated $Q/d_{2537} = 8.7 \times 10^{18}$ $h\nu$/ml pH 8.2	A	61	8.6	14.1
	B	79	9.0	14.4
Bovine serum albumin irradiated $Q/d_{2537} = 8.0 \times 10^{18}$ $h\nu$/ml pH 6.2	C	52	7.6	11.3
	D	74	7.3	11.0
	E	37	7.0	10.6

A and C unfractionated.
B = First fraction (lowest adsorption) from frontal analysis on active carbon.
D and C = Top and bottom fractions from run in the Spinco centrifuge.
The total concentration was determined in a micro interferometer.

present author found that the presence of sodium caprylate had no influence on the irradiation effects of the spectrum at pH 6.2, see Fig. 20, an opinion shared by Giese *et al.* (70). It is not unlikely, however, that aggregation is less pronounced in the sodium caprylate medium and that decrease or absence of light scattering is the explanation in the case of Luck *et al.* This is also in keeping with some results obtained at pH 5.8 when perfluoro-octanoic acid was present, see Fig. 20. The increase in extinction was in this case that usually encountered at pH 6.2 whereas the normally very pronounced light scattering at pH 5.8, which is due to the formation of large aggregates, was absent, compare also page 96 and 97.

It is interesting to compare the results with those for serum albumin irradiated with ionizing irradiation where also aggregation occurs but where the action is indirect and caused by radicals produced in the solvent. Barron and Finkelstein (87) noticed the increase in the maximum and in the minimum and explain it as due to changes in the aromatic groups. On the other hand Rosen (88) is of the opinion that most of the changes are due to light scattering. However, he uses $n = 4$ for correcting the spectrum and remarks himself that the correction values are uncertain. A lower n value would make the changes due to light scattering considerably smaller. Rosen and Boman (18) when fractionating irradiated serum albumin chromatographically found that the fraction that in the ultracentrifuge was shown to be unchanged serum albumin also had unchanged light absorption. This result is quite in agreement with the calculation by Rosen who finds that every molecule acted upon by a radical must form part of an aggregate. As mentioned above after irradiation with ultraviolet light molecules with unchanged molecular weight show changed absorption spectra.

Conclusions

From the experience with the above-mentioned irradiated protein solutions the following conclusions may be drawn. The spectra show an increase in extinction over the whole spectrum also for $\lambda > 3000$ Å and the difference between the original

maximum and minimum disappears. At very large Q/d values a decrease in the extinction at the former maximum may also be observed. As large aggregates appear light scattering increases the extinction. There is no exact correlation between the changes in the protein spectrum and that of the corresponding aromatic amino acid mixture, but the former seem to be larger. The effect is very strong for serum albumin but is not so pronounced for hemocyanin. Energy transfer is here a possible explanation and may involve both aromatic groups and groups with normally low absorption at 2537 Å. For the irradiation doses used in chapter VI the change in light absorption for hemocyanin is not large enough to effect markedly the distribution of absorbed energy within the molecule. This may however be the case for serum albumin. The changes in the absorption spectrum do not appear to be connected with the changes in the molecular weight and it is suggested that they occur evenly for the whole material.

Chapter IV

Determination of quantum yields

The absorption of ultraviolet light by such a complex molecule as a protein in aqueous solution will cause a number of primary photochemical reactions. It has already been mentioned in chapter III that the energy at λ 2537 Å is sufficient to break any single bond in the protein molecules.

If no secondary reaction follows, the quantum yields for the different reactions are easily calculated. On the other hand if such reactions occur immediately, the quantum yield, on the basis of the effects observed after irradiation, will be a measure both of the direct and indirect effects of light. Investigations of the products formed in the primary reaction are in this case very difficult, especially when the reaction takes place at room temperature.

For the primary photochemical reaction analogous considerations as described by Förster (89) may be used. Knowledge of the effects of the action of ionizing radiation on synthetic polymers and proteins (2) is also of help when possible mechanisms are discussed.

The following cases are of importance (A) Excitation. (B) Splitting into two stable molecules. (C) Splitting into one large macroradical and one small radical that can easily diffuse away thus avoiding recombination.

The possibility that splitting occurs into two large macroradicals or two other reactive species is judged to be less likely as the cage of solvent molecules surrounding the radicals makes the probability of recombination great (cage effect or Franck-Rabinowitch effect).

The main secondary reactions will be deactivation, aggregation, and further splitting. In case (B) there are no secondary reactions involving the original molecules.

Unchanged or intact molecules are hereinafter taken to mean unchanged with respect to the specific property that is investigated.

General case

We shall now consider the general case where secondary reactions may also interfere with the concentration of unchanged material and where the light absorption changes at the wavelength used for irradiation.

However, the influence of light scattering on the absorption of different protein components must first be discussed.

For a system of two components, 1 and 2, where 1 exhibits both absorption and scattering and 2 absorption only, the decrease dI, in the incident light intensity, I, in a layer of thickness dl, is given by Beer's law as $dI = 2.303 \times I(c_1(E_{a1} + E_s) + c_2 E_{a2})\, dl$. E_{a1} and E_{a2} are the absorption coefficients, E_{s1} the scattering coefficient and c_1 and c_2 the concentrations.

The ratio between the light absorbed by the components is $(dI_A)_1/(dI_A)_2 = c_1 E_{a1}/c_2 E_{a2}$.

Reabsorption of the scattered light also occurs in the above-mentioned ratio, which thus is valid independently of light scattering.

We assume that we deal with a system that at a given moment, t, after irradiation has started, contains i different kinds of molecules, (i varying with time), with a concentration of N_j molecules/ml ($j = 1, \ldots, i$); γ different kinds of molecules have the same molecular weight M. The corresponding absorption coefficient at 1 per cent and at the wavelength in question is $E_{a,j}$. The total concentration is C per cent and the absorption coefficient, at 1 per cent, for the whole solution E_a. During a time interval, dt, at the time t, dQ quanta are absorbed per ml solution.

If ϱ is the density of the solution and $\mathcal{N}$ Avogadro's constant we get according to Beer's law (dQ can be used instead of dI with good approximation) for the number of quanta absorbed during dt, by the jth kind of molecules with molecular weight M_j

$$dQ \frac{N_j 100 M_j E_{a,j}}{\mathcal{N} \varrho C E_a}.$$

It should be noticed that Q is measured in quanta per ml which seemed to be the natural unit to use as the volumes were kept constant in the cuvettes, see chapter II.

By definition the quantum yield ϕ_j for the jth kind is the number of changed molecules, dN_j, divided by the number of quanta absorbed by the N_j molecules. Thus

$$\phi_j = - \frac{dN_j \mathcal{N} \varrho C E_a}{dQ\, N_j 100 M_j E_{a,j}}. \tag{1}$$

If we are only observing a change that affects the γ kind of molecules with molecular weight M, the total number of observed "unchanged" molecules is $N = \sum_{j=1}^{\gamma} N_j$ and $dN = \sum_{j=1}^{\gamma} dN_j$

$$dN = - \frac{dQ\, 100 M}{\mathcal{N} \varrho C E_a} \sum_{j=1}^{\gamma} N_j E_{a,j} \phi_j.$$

If we define the mean values $\bar{E}_{a,j}$ and $\bar{\phi}_j$ by

$$\sum_{j=1}^{\gamma} N_j E_{a,j} \phi_j = N \bar{E}_{a,j} \bar{\phi}_j$$

we get

$$\bar{\phi}_j = -\frac{dN \mathfrak{N} \varrho C E_a}{N dQ 100 M \bar{E}_{a,j}} . \tag{2}$$

In order to calculate $\bar{\phi}_j$, $\bar{E}_{a,j}$ has to be known. If no change in light absorption occurs during irradiation we have of course $E_a = E_{a,j} = \text{const.}$ and $\bar{\phi}_j$ is easily calculated. Another simple case is when the changed molecules have lost all their light absorption. It has been pointed out by Hausser (90) that if $E_{a,j} = \text{const.}$ but E_a variable the molecules of the jth kind will still absorb the same number of quanta as at the beginning as long as the optical density is low enough to be directly proportional to the amount of light absorbed.

For the proteins used in this investigation it was found in chapter III that the change in light absorption very likely occurred evenly over the whole material, thus making the number of chromophores per gram material nearly the same for all components. We can then put $E_a = \bar{E}_{a,j}$ and the expression for $\bar{\phi}_j$ becomes

$$\bar{\phi}_j = -\frac{K C d N}{N d Q} \quad \text{where } K = \frac{\mathfrak{N} \varrho}{100 M} . \tag{3}$$

If $ln\ N/N^0$ or $ln\ (c/c^0)$, (concentration in per cent) is plotted against Q/C (c^0 is not necessarily $= C$, $\gamma \neq i$ at $t = 0$), we get $\bar{\phi}_j$ from the slope at any given point.

If the quantum yield is larger than zero immediately after the irradiation has started, only one effective quantum (one-hit process) is needed to bring about a change. If a straight line is observed, $\bar{\phi}_j$ is constant and the quantum yield is the same for all the molecules in question. A one-hit process is on the other hand not always characterized by a straight line; if the quantum yield changes with time or from molecule to molecule a curved line is obtained (see also page 44).

If ϕ_j is zero at first and then shows a marked increase, more than one effective quantum (an n-hit process) is needed to bring about a change. However, the value of n cannot be determined accurately under the above conditions as the secondary reactions will complicate matters considerably.

If induction periods are present it may be difficult to determine if a one-hit process or a multi-hit process occurs, especially if only the first part of the curve is available. More precise information can only be obtained from the above mentioned curves when the molecules formed do not react with intact material.

Constant absorption coefficient and no influence by secondary reactions

We will now treat the simple case where the absorption coefficient at the wavelength used for irradiation does not change during irradiation and the secondary reactions do not involve the intact molecules. The probability for one of the intact molecules to absorb a light quantum will then be the same during the whole irradiation. As already mentioned it is only necessary that the intact molecules have unchanged absorption if very low optical densities are used. For the deduction of the quantum yields we can then use a treatment—"the target theory"—similar to

that employed by Timoféeff-Ressovsky and Zimmer (91) and Lea (92) for ionizing irradiation and ultraviolet light.

We assume that a solution in a non-absorbing solvent, containing N^0 identical molecules of a given solute initially, after having absorbed totally Q quanta, has N solute molecules left intact. We assume further that the probability that an absorbed quantum will bring about a certain permanent internal change, necessary for the final observed change, is ϕ. If several such changes are needed, ϕ is presumed to be constant and independent of the fact that one or several internal changes already have occurred in the molecule. The average amount of absorbed quanta per intact molecule will be Q/N^0 which is denoted by D. The average number of effectively absorbed quanta or internal changes per molecule will then be $D\phi$.

The absorption will follow statistical laws and in this case the Poisson distribution may be applied (the quanta are absorbed independently of each other) and ϕ is small.

The probability that exactly n internal changes has occurred in a molecule when $D\phi$ are expected is

$$\frac{(D\phi)^n e^{-D\phi}}{n!}. \tag{4}$$

If only one internal change (one-hit process) is needed to change the molecule we have $(n = 0)$

$$N = N^0 e^{-D\phi}. \tag{5}$$

If n internal changes (n-hit process) are needed, we have to make a summation of all the molecules that have suffered not more than $n - 1$ internal changes to get N

$$N = N^0 e^{-D\phi} \sum_{k=0}^{n-1} \frac{(D\phi)^k}{k!}. \tag{6}$$

For the identification of the curves, $\ln N/N^0$ is conveniently plotted against D, or for normalisation, against $D/D_{\frac{1}{2}}$ where $D_{\frac{1}{2}}$ is the value corresponding to $N/N^0 = \frac{1}{2}$ (see Fig. 22). Plotting against D gives of course the same kind of curves as those above with Q/C.

From the expression for the "one-hit" process (Eq. (5)) a straight line is derived where ϕ, the quantum yield, is obtained from the slope. For the n-hit process curved lines are obtained. The value of n may be calculated from certain formulas (91).

The case where the internal changes in a multi-hit process have to occur within a certain time has been dealt with by Lea (91, 92) for certain special conditions. The quantum yield will then vary with the intensity.

Especially for biological material some molecules may have different ϕ-values. We assume that there are i different kinds of molecules with ϕ-values $= \phi_j$ $(j = 1, \ldots, i)$.

For a one-hit process we then have $dN_j = -\phi_j\ (dQ\ N_j/N^0)$, and get $\Sigma dN_j = dN = -(dQ/N^0)\ \Sigma N_j\phi_j$, where $\Sigma N_j = N$. We then put $dN = (dQ/N^0)\ N\ \bar{\phi}$ where $\bar{\phi}$ represents a mean value which varies with Q/N^0.

Fig. 22 shows the corresponding curve. It is, however, necessary that about half the material has a ϕ-value differing ten times from that of the other half to get a

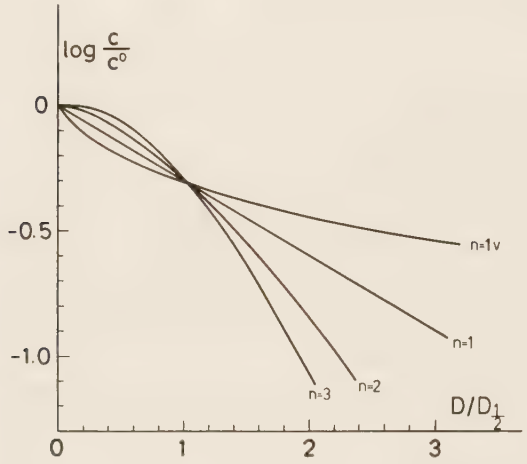


Fig. 22. The decrease in the amount of intact molecules upon irradiation for $n = 1$, 2 and 3 and for a one-hit process with variable quantum yield ($n = 1\ v$).

considerable deviation from a straight line. The same kind of curve is obtained in a one-hit process where the extinction coefficient changes during irradiation, see above. For a "multi-hit" process with variable ϕ-value the curve will be characterized by a lower n-value than it actually has.

Influence of secondary reactions

Among secondary reactions involving the original molecules (cases (A) and (C)), chain polymerization is judged to be of small importance as the large radicals with their many aromatic groups will have great resonance stability and low reactivity (2). The action of small radicals formed will probably also play an unimportant role as the concentration as well as the efficiency of such action is often low.

The secondary reactions that may interfere with the molecules unchanged by irradiation are thus the reaction of a denatured or excited molecule or a macro-radical with the intact material.

The molecules of the original kind are called P_{M_1} with molecular weight M_1. After irradiation has proceeded for some time we have i different kinds of molecules. For the original kind we have the following simplified reaction scheme

$$\text{(a)} \quad P_{M_1} \xrightarrow{h\nu} P^*_{M_1}$$

$$\text{(b)} \quad P^*_{M_1} + P^*_{M_j} \rightarrow P_{M_1+M_j} \qquad j = 1, 2, \ldots, i$$

$$\text{(c)} \quad P^*_{M_1} + P_{M_j} \rightarrow P_{M_1+M_j} + (R)$$

$$\text{(d)} \quad P^*_{M_1} + S \rightarrow P_{M_1} + (R)$$

where the star designates the reactive species and R possible small radicals with molecular weight negligible compared to M_j, S is the solvent.

It is immediately seen that if reaction (b) is dominant no interaction with intact material occurs; if reaction (d) is dominant there occurs no changes in the molecular weight. On the other hand if the shape of the molecule is altered a change in the

sedimentation constant may be observed in the ultracentrifuge. The relevant case in this connection is when reaction (c) is dominant or comparable to reactions (b) and (d). The progress of the complete reaction will then be very complex leading to a wide distribution of molecular weights.

However, it is easily seen that during the first part of the reaction when the majority of molecules are unchanged P_{M_1}, reaction (c) will only be significant for $M_j \quad M_1$ and when this reaction is dominant, *two* molecules P_{M_1} will disappear for each effective quantum hit.

A determination of the quantum yield ϕ from the slope of the curve $\ln c_j c^0$ versus $Q \; C'$ (cf. page 42), where c denotes the concentration of unchanged molecules P_{M_1}, will then give a value at $t = 0$ (from the initial slope) which is twice the value for the quantum yield ϕ_{M_1} for the primary reaction (a),

$$\bar{\phi}^0 = 2 \; \phi_{M_1}. \tag{7}$$

For the rather unlikely case of the occurence of splitting into two large reactive species we can discuss the simplified reaction scheme in the same way

$$\begin{aligned}
&\text{(e)} \quad P_{M_1} \xrightarrow{h\nu} P^*_{M_j} + P^*_{M_1 - M_j} \\
&\text{(f)} \quad P^*_{M_j} + P^*_{M_k} \rightarrow P_{M_j + M_k} \\
&\text{(g)} \quad P^*_{M_j} + P_{M_k} \rightarrow P_{M_j + M_k} + (R) \qquad j = 1, 2, \ldots, i \\
&\text{(h)} \quad P^*_{M_j} + S \rightarrow P_{M_j} + (R)
\end{aligned}$$

It is seen directly that a dominating reverse reaction in the case of the reaction (e) will cause the actual concentration of P^* to be proportional to the square root of the light intensity (13). It is also seen that if the formation of aggregates, which in this case can be both smaller and larger than M_1, is dominated by reaction (g) each effective quantum hit will split one molecule in two parts each of which will aggregate with an intact molecule. Thus *three* molecules P_{M_1} will disappear for each effective quantum hit during the first part of the reaction when the concentration of P_{M_1} is dominating.

In complete analogy with the previous case we then get

$$\bar{\phi}^0 = 3 \; \phi_{M_1} \tag{8}$$

where ϕ_M is the quantum yield for reaction (e) and ϕ^0 the observed quantum yield from the initial slope of the curve.

Quantum yields from osmotic pressure measurements combined with ultracentrifugation data

If from ultracentrifugations it has been ascertained whether splitting or aggregation is the principal reaction, determination of the number average molecular weights from osmotic pressure measurements can give valuable additional information on the primary photochemical quantum yield.

If we denote the number of molecules per ml at the start and at the time t by N^0 and N respectively and the original molecular weight by M_1, we have for the number average molecular weight at the time t, $M_n = M_1 N^0 / N$.

This expression takes a very simple form for the two cases for which Eqs. (7) and (8) respectively are valid. In the first case one molecule disappears for each effective quantum hit since two molecules P_{M_1} are used up to make one aggregate. As a dose Q gives $Q\ \phi_{M_1}$ effective hits we get

$$M_n = \frac{M_1 N^0}{N_0 - Q\,\phi_{M_1}} \quad \text{and} \quad \frac{d\,\frac{1}{M_n}}{dQ} = -\frac{\phi_{M_1}}{M_1 N^0}\,.$$

For the second case we find analogously that three molecules P_{M_1} are used up to make two aggregates and so one molecule is lost for each effective quantum hit. We thus get the same expression as before.

Thus in principle it is possible to determine both ϕ_{M_1} and $\bar{\phi}^0$ from measurements of osmotic pressure and sedimentation. In practice, however, it is often difficult to get good osmotic values for the aggregated solutions.

It should be pointed out that in cases where the aggregation rate is proportional to the square root of the light intensity this will show up as ϕ values variable with the intensity. When the quantum yield is independent of the intensity this implies that in a splitting reaction the reverse reaction is negligible.

Quantum yields determined for proteins

Determinations of quantum yields have mostly been made for irradiated enzymes where the decline in enzyme activity has been measured. From experiments with a great many enzymes McLaren *et al.* have found that in all cases a one-hit process is involved. In one case the intensity has been varied 10^5 times (93). The quantum yield varies with factors such as pH and salt concentration, but if an average value is taken for each protein it seems to be inversely proportional to the molecular weight (66). For pepsin ($M = 36{,}000$) the quantum yield is given as $\sim 2 \times 10^{-3}$ (82, 93).

As destruction of enzyme activity generally occurs when the first stages of denaturation takes place, that is after unfolding and breakage of the weak bonds near the surface, it is not surprising that fewer quanta are needed in the case of a small molecule where this surface layer contains proportionally a larger amount of chromophores than in a molecule with larger molecular weight if the shape is approximately the same.

The inverse relation of quantum yield to molecular weight also means, assuming on the average the same number of chromophores per gram substance, that irrespective of the molecular weight of the enzyme the chromophores have absorbed on the average the same number of quanta when an inactivation occurs.

Shugar has also irradiated enzymes and found evidence of a one-hit process (94, 95). However, in several cases he has found that the quantum yield decreases with increasing concentration and he discusses the possible reasons for this at some length (95). The most likely reason for a decreased quantum yield at increasing concentrations for a one-hit process is, however, as pointed out by McLaren and Pearson (82), that the stirring might be insufficient; the discrepancy between the quantum yield for pepsin determined by different workers is explained in this way by the last mentioned authors.

Changes in light absorption are in themselves no measure of the inactivation, but as the reactions leading to changed light absorption and those causing inactiva-

tion run parallel they may more or less indirectly be used to show how far the reaction has advanced.

The destruction of the enzyme activity of pepsin after irradiation has lately been ascribed to the destruction of the tyrosyl groups (96). It is, however, known that for instance urea destroys the enzyme activity without any changes occuring in the spectrum (82) and we have thus a case where an easily detected effect (change in light absorption) has been made the cause of another easily detected effect (change in enzyme activity).

Kleczowski (8), has compared the decrease in enzyme activity and in stability—measured as stability when exposed to 37°C—of chymotrypsin and found that the former decreases more slowly than the latter.

There is no necessity that quantum yields measured from the changes in molecular weight should be of the same order of magnitude as those for changes in enzyme activity.

It would be of great value to get the total quantum yield of the light absorbed in a molecule by means of calorimetric measurements and thus to ascertain how much of the energy absorbed is used up inside the molecule.

Chapter V

Determination of sedimentation constants and concentrations in concentration dependent sedimentation

Ultracentrifugation was the main method used for the study of the irradiated protein solutions. This method is too well known to need any further description. For the general theory and for technical details the reader is referred to the monograph by Svedberg and Pedersen (97). Lamm's scale method was used exclusively (98). A recent review by Williams (99) also gives good general information.

The irradiation of *Helix pomatia* hemocyanin and bovine serum albumin produces multi-component systems. From the ultracentrifuge diagrams the concentration of material unchanged as to sedimentation behaviour is obtained. As the sedimentation is concentration dependent for both the above proteins it is important to know to what extent this influences the measured concentrations and sedimentation constants.

One-component systems

For the sedimentation in a one-component system (one sedimenting solute in the solvent) the well-known equation holds

$$\frac{dx}{\omega^2 x\, dt} = s^t \tag{1}$$

where x is the distance from the centre of rotation, ω the angular velocity, s^t the sedimentation constant at the concentration c^t which is the concentration in the cell at the time t. We are assuming here that c^t at any given moment is constant below the sedimenting boundary, an assumption which is valid for water solutions where the effect of the hydrostatic pressure is negligible. Kinell (100) has treated the

more general case. Due to the sector shape of the cell the concentration will change and hence the sedimentation constant will also change continuously.

In the ultracentrifuge diagram, the concentration gradient dc/dx in the cell is plotted against x and the concentration $c^t = \int (dc/dx)\, dx$ is immediately obtained by planimetry. We want, however, to know the original concentration c^0. Svedberg and Rinde (97) derived the expression for sedimentation independent of the concentration and got

$$c^0 = c^t \left(\frac{x^t}{x^0}\right)^2 \tag{2}$$

where x^t is the distance from the centre of the rotor to the boundary at the time t, x^0 that at zero time. Trautman and Schumaker (101) have shown that this expression is also valid for concentration dependent sedimentation; they assume constant c^t below the boundary. Kinell (100) and Eriksson (102) have treated the case when a concentration gradient is present below the sedimenting boundary.

The author has also deduced Eq. (2) for concentration dependent sedimentation assuming no concentration gradient below the boundary. The following considerations were made.

At the start of the sedimentation ($t = 0$) we have $x = x^0$ and $c = c^0$; at the time t, $x = x^t$ and $c = c^t$. θ is the sector angle and l the length of the cell.

Figure 23 shows the cell in perspective, dotted lines complete the sector.

Two stationary cylindrical surfaces are placed below the boundary at constant distance a and b respectively from the centre of rotation. The change in concentration dc^t in the volume between these surfaces in the time interval dt is equal to the difference between the amount of material that has entered the volume and that which has left it, divided by the volume

$$\therefore\ dc^t = \frac{\dfrac{(dx)_a \pi\, \theta\, a\, l\, c^t}{180} - \dfrac{(dx)_b \pi\, \theta\, b\, l\, c^t}{180}}{\theta \pi \dfrac{(b^2 - a^2)\, l}{360}}$$

$(dx)_a$ and $(dx)_b$ is the distance that a molecule at the distance a and b respectively from the centre of rotation sediments during the time dt. They are obtained from

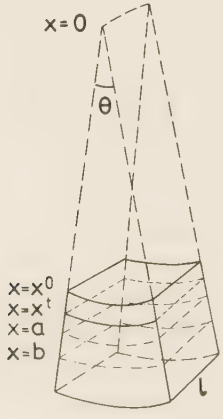


Fig. 23. Ultracentrifuge cell.

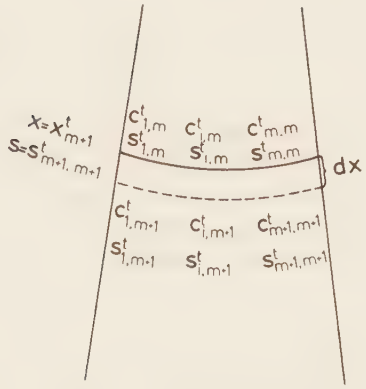


Fig. 24. The $(m+1)$st boundary in the ultracentrifuge cell.

Eq. (1) for $x = a$ and b respectively. When these expressions are put into the above equation for dc^t, it becomes

$$-dc^t = 2c^t\omega^2 s^t dt.$$

Inserting the value of s^t from Eq. (1) we have

$$\therefore\ -dc^t = 2c^t dx/x$$

which after integration yields

$$c^0 - c^t\left(\frac{x^t}{x^0}\right)^2.$$

As the boundary in practice is not sharp, it is necessary to calculate the position $\bar{x}^t$ of the fictive sharp boundary in order to be able to use Eq. (2). The value for x^t is obtained by using the law of conservation of mass as above, and if x^γ is the distance from the rotor axis to the region where the concentration becomes constant, i.e. c^t, we get

$$c^t[(x^\gamma)^2 - (\bar{x^t})^2] = \int_{x^0}^{x^\gamma}[(x^\gamma)^2 - x^2]\frac{dc^t}{dx}dx$$

$$\bar{x^t} = \sqrt{\frac{\int_{x^0}^{x^\gamma} x^2\frac{dc^t}{dx}dx}{c^t}} \qquad (3)$$

which is the square root of the second moment of the gradient curve. This expression has been given in an extensive investigation by Goldberg (103). The x-value corresponding to the maximum value of dc^t/dt is often used in practice for x^t and is a good approximation if the peak is not too wide and unsymmetrical.

From the ultracentrifuge diagram we get a time mean value for the sedimentation constant

$$\bar{s^t} = \frac{\ln \frac{x^{t_2}}{x^{t_1}}}{\omega^2 (t_2 - t_1)} = \frac{x^{t_2} - x^{t_1}}{\omega^2 x^m (t_2 - t_1)} \tag{4}$$

where

$$\tfrac{1}{2} (x^{t_2} + x^{t_1}) = x^m.$$

The corresponding time mean value for the concentration

$$c^t = \frac{\int_{t_1}^{t_2} c^t \, dt}{t_2 - t_1} \tag{5}$$

can be calculated if the expression for the variation of s^t with c^t is known.

In the short concentration interval in a cell of normal dimensions, $0.7\, c^0 < c^t < c^0$, we can in most cases use the following expression for the concentration dependence of the sedimentation constant

$$s^0 (1 + k c^0) = s^t (1 + k c^t). \tag{6}$$

Under the assumption that Eq. (6) holds also for s^t and c^t we will calculate $\bar{c^t}$. We get $\bar{c^t}$ directly by integrating Eq. (5) with the values for c^t and dt from Eq. (1) and (2) inserted and using Eq. (4) and (6). We finally get

$$\bar{c^t} (1 + k \bar{c^t}) = c^0 \left(\frac{x^0 x^m}{x^{t_1} x^{t_2}} \right)^2 \left[1 + \tfrac{1}{2} k c^0 (x^0)^2 \frac{(x^{t_1})^2 + (x^{t_2})^2}{(x^{t_1} x^{t_2})^2} \right]$$

and as $(x^m)^2 \cong x^{t_1} x^{t_2}$

$$\bar{c^t} (1 + k \bar{c^t}) = c^0 \frac{(x^0)^2}{x^{t_1} x^{t_2}} \left[1 + k c^0 \left(\frac{x^0}{x^m} \right)^2 \right] \tag{7 a}$$

and

$$\bar{c^t} = c^0 \frac{(x^0)^2}{x^{t_1} x^{t_2}} = c^0 \left(\frac{x^0}{x^m} \right)^2. \tag{7 b}$$

It is thus seen that, with good approximation, the concentration in the midpoint x^m corresponds to $\bar{s^t}$.

The same expression for $\bar{c^t}$ is also obtained in the following way. The values for s^t and c^t from Eq. (1) and (2) are inserted in Eq. (6) and we get

$$s^0 (1 + k c^0) = \frac{d x^t}{\omega^2 x^t \, dt} \left[1 + k c^0 \left(\frac{x^0}{x^t} \right)^2 \right],$$

and upon integration and using Eq. (4) this becomes

$$s^0 (1 + k c^0) = \frac{(x^{t_2} - x^{t_1})}{(t_2 - t_1) \omega^2 x^m} \left[1 + k c^0 \left(\frac{x^0 x^m}{x^{t_1} x^{t_2}} \right)^2 \right]$$

which again gives Eq. (7 b).

It should be noticed that the corresponding distance mean value for the concentration is in practice very nearly the same as the time mean value

$$\bar{c}_x = \frac{\int_{x^{t_1}}^{x^{t_2}} c^0 \left(\frac{x^0}{x}\right)^2 dx}{x^{t_2} - x^{t_1}},$$

$$\therefore \bar{c}_x = c^0 \frac{(x^0)^2}{x^{t_1} x^{t_2}} = c^0 \left(\frac{x^0}{x^m}\right)^2. \qquad (7\text{ c})$$

Jullander (104) has discussed the strongly concentration dependent sedimentation of nitrocelluloses and has also earlier (unpublished) shown graphically the variation of the sedimentation constant during a single run. The sedimentation constants were calculated from exposures at very short time intervals. Alberty (105) has also discussed the best way of deriving the sedimentation constants corresponding to the concentration in the ultracentrifuge cell at the start.

n-Component systems

For an n-component system where the sedimentation of the different components affect each other, the situation becomes more complicated. Anomalies in the sedimentation of mixtures had long been observed but were first explained by Johnston and Ogston (106), who treated the case of a two-component mixture in a cylindrical cell. Similar phenomena have been treated for electrophoresis and for frontal analysis in the field of chromatography by for instance Svensson (107) and Claesson (108) respectively. In our case (see chapter VI, 1 and 2), where we have to discuss the concentration change in paucidisperse systems from sedimentation diagrams, it would be very convenient to be able to calculate the magnitude of the Johnston-Ogston effect directly from the concentration dependence of the sedimentation constants.

The following treatment is similar to those in the above-mentioned works. We consider the sedimentation of a system of n components (solutes) of initial concentration c_i^0 ($i = 1,2, \ldots, n$) forming n sharp boundaries at the position x_i^t in the cell at the time t. No concentration gradient is supposed to exist between the boundaries. We denote by $s_{i,m}^t$ the sedimentation constant for the ith component when it sediments in the volume between the mth and the $(m+1)$st boundary. The concentration of the components present in this volume are $c_{1,m}^t, c_{2,m}^t, \ldots, c_{m,m}^t$. The sedimentation constant $s_{i,m}^t$ is dependent on all these concentrations. Component 1 is the slowest and component n the fastest moving one. The components are assumed to be in equilibrium with each other; in the case of a sector shaped cell a continuous change from one state of equilibrium to another takes place.

We consider the sedimentation of the ith component on both sides of the $(m+1)$th boundary (see Fig. 24) between the times t and $t + dt$. In the time dt this boundary, which indicates the sedimentation of the $(m+1)$st component, moves a distance dx which according to Eq. (1) is

$$dx = \omega^2 x_{m+1}^t s_{m+1,\,m+1}^t dt.$$

The corresponding volume V is, if we disregard the sector shape of this small volume and assume a constant cross-sectional area of A, $V = A\,dx$. The concentration in this volume at the time t is $c^t_{i,\,m+1}$, at the time $t+dt$, $c^{t+dt}_{i,\,m}$ which for dt small is $c^t_{i,\,m}$. The difference in the amount of the ith component in the volume V is then $A\,\omega^2\,x^t_{m+1}\,s^t_{m+1,\,m+1}(c^t_{i,\,m} - c^t_{i,\,m+1})\,dt$, which is also the difference between the amount of material entering the volume and leaving it, that is

$$A\,\omega^2\,x^t_{m+1}\,s^t_{i,\,m}\,c^t_{i,\,m}\,dt - A\,\omega^2\,x^t_{m+1}\,s^t_{i,\,m+1}\,c^t_{i,\,m+1}\,dt$$

$$\therefore\ c^t_{i,\,m+1} = c^t_{i,\,m}\,\frac{1 - \dfrac{s^t_{i,\,m}}{s^t_{m+1,\,m+1}}}{1 - \dfrac{s^t_{i,\,m+1}}{s^t_{m+1,\,m+1}}} \qquad \begin{matrix} i = 1,\ \ldots,\ n, \\ m = 1,\ \ldots,\ n. \end{matrix} \tag{8 a}$$

It was shown by Claesson (109) that an analogous expression for the concentrations in frontal adsorption analysis could be put in a particularly simple form by using a suitable generalization of a formula equivalent to Eq. (6). We will introduce this generalization which here will be

$$s^t_{i,\,m} = s_{i,\,0}\,f_{i,\,m}\,(c^t_{1,\,m},\ c^t_{2,\,m},\ \ldots,\ c^t_{m,\,m}) \quad (m = 1,\ \ldots,\ n) \tag{9}$$

where the function $f_{i,\,m}$ is the same for all i. We always choose $f_{i,\,m} = 1$ for $\sum_{i=1}^{m} c_{i,\,m} = 0$, the constant $s_{i,\,0}$ is thus the sedimentation constant for component i at infinite dilution. The function $f_{i,\,m}$ can obviously be chosen within rather wide limits to fit given experimental data. To get Eq. (6) we should choose $f = 1/(1 + k\,c)$.

From Eq. (9) we get

$$\frac{s^t_{i,\,m}}{s^t_{m,\,m}} = \frac{s_{i,\,0}}{s_{m,\,0}} \quad \text{and} \quad \frac{s^t_{i,\,m+1}}{s^t_{m+1,\,m+1}} = \frac{s_{i,\,0}}{s_{m+1,\,0}}\,. \tag{10}$$

If we multiply the second term in the numerator of Eq. (8 a) with $s^t_{m,\,m}/s^t_{m,\,m}$ and use Eq. (10), we have (108)

$$c^t_{i,\,m+1} = c^t_{i,\,m}\,\frac{1 - \dfrac{s_{i,\,0}}{s_{m,\,0}}\,\dfrac{s^t_{m,\,m}}{s^t_{m+1,\,m+1}}}{1 - \dfrac{s_{i,\,0}}{s_{m+1,\,0}}} \qquad \begin{matrix} i = 1,\ \ldots,\ n, \\ m = 1,\ \ldots,\ n. \end{matrix} \tag{8 b}$$

This is a set of recursion formulas by means of which it is possible to calculate all concentrations at a given time t from the observable values $s^t_{j,\,j}\,(j = 1,\ \ldots,\ n)$ and $\sum_{i=1}^{m} c^t_{i,\,m}\ (m = 1,\ \ldots,\ n)$ and the values of $s_{i,\,0}$ which must be known The exposures are taken at short time intervals so that the concentration does not change significantly. We have regarded the cross section of the cell as constant and this may be valid if we do not integrate over any appreciable length of time but take the observations of the sedimentation constant during a short period as indicated above.

We wish to know not only $c^t_{i,m}$ but $c^0_i = c^0_{i,n}$. Below the last, nth boundary, component i has the concentration $c^t_{i,n}$. We consider now the fictive boundary at a distance $x^t_{i,f}$ from the centre of rotation which would mark the sedimentation of the ith component when sedimentating alone in the same way as it does below the nth boundary. This value $x^t_{i,f}$ is obviously the proper value to use in Eq. (2) for calculating c^0_i from $c^t_{i,n}$. Trautman and Schumaker (101) have pointed out that the value for $x^t_{i,f}$ is defined by Goldberg's expression Eq. (3). As there is a concentration change for the component i at all the boundaries i, $i+1$, ..., n in the cell, this expression is difficult to evaluate in practice. However, if Eq. (9) is applied we get a much simpler expression for $x^t_{i,f}$.

According to Eq. (1) we have

$$\frac{d\,x^t_{i,f}}{x^t_{i,f}} = s^t_{i,n}\,\omega^2\,dt \tag{11 a}$$

and for the nth component

$$\frac{d\,x^t_n}{x^t_n} = s^t_{n,n}\,\omega^2\,dt. \tag{11 b}$$

Dividing Eq. (11 a) with (11 b) and using Eq. (10) we get after integration

$$\ln\frac{x^t_i}{x^0} = \frac{s_{i,0}}{s_{n,0}}\ln\frac{x^t_n}{x^0} \quad \text{or} \quad \frac{x^t_i}{x^0} = \left(\frac{x^t_n}{x^0}\right)^{s_{i,0}/s_{n,0}}$$

where x^t_n is the position of the lowest boundary in the ultracentrifuge cell at the time t. For the nth component we have directly from Eq. (2)

$$c^0_n = c^t_{n,n}\left(\frac{x^t_n}{x^0}\right)^2.$$

Thus

$$c^0_i = c^t_{i,n}\left(\frac{x^t_n}{x^0}\right)^{2\,s_{i,0}/s_{n,0}}. \tag{12}$$

Harrington and Schachman have discussed different sedimentation behaviours (110) and Schachman *et al.* (111) have derived an expression for a two-component system in a different way. Their equation becomes identical with the above if Eq. (10) is applied.

Two-component systems

For a two-component system Eq. (8 a) becomes

$$c^t_{1,2} = c^t_{1,1}\frac{1-\dfrac{s^t_{1,1}}{s^t_{2,2}}}{1-\dfrac{s^t_{1,2}}{s^t_{2,2}}} = c_{1,1}\,\frac{s^t_{2,2}-s^t_{1,1}}{s^t_{2,2}-s^t_{1,2}}$$

which is the same as that of Johnston and Ogston (106). Eq. (8 b) gives

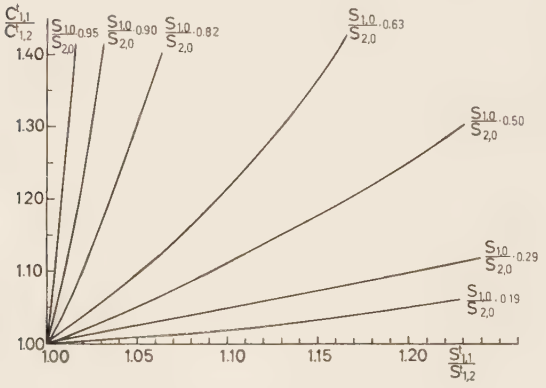


Fig. 25. Concentration ratio $c^t_{1,1}/c^t_{1,2}$ as a function of $s^t_{1,1}/s^t_{1,2}$.

$$c^t_{1,2} = c^t_{1,1}\frac{1-\dfrac{s^t_{1,1}}{s^t_{2,2}}}{1-\dfrac{s_{1,0}}{s_{2,0}}} = c^t_{1,1}\frac{1-\dfrac{s^t_{1,1}\,s_{1,0}}{s^t_{1,2}\,s_{2,0}}}{1-\dfrac{s_{1,0}}{s_{2,0}}}.$$

The variation of $c^t_{1,1}/c^t_{1,2}$ with $s^t_{1,1}/s^t_{1,2}$ for given $s_{1,0}/s_{2,0}$ is seen in Fig. 25.

For the concentrations of a two-component system we have, compare Eq. (12),

$$c^0_2 = c^t_{2,2}\left(\frac{x^t_2}{x^0}\right)^2 \quad \text{and} \quad c^0_1 = c^t_{1,2}\left(\frac{x^t_2}{x^0}\right)^{2\,s_{1,0}/s_{2,0}}.$$

It might be of interest to mention that it is possible to determine the composition of a two-component system for which Eq. (10) is valid by means of two ultracentrifugations if in one of these a known amount of component 1 is added to the original mixture. (Pure component 1 may be obtained from a run in a separation cell.) We have for run number one

$$c^{t_1}_{1,2} = c^{t_1}_{1,1}\frac{1-\dfrac{s^{t_1}_{1,1}}{s^{t_1}_{2,2}}}{1-\dfrac{s_{1,0}}{s_{2,0}}} = \frac{c^0_1}{\left(\dfrac{x^{t_1}_2}{x_0}\right)^{2\,s_{1,0}/s_{2,0}}},$$

for run number two

$$c^{\prime t_2}_{1,2} = c^{\prime t_2}_{1,1}\frac{1-\dfrac{s^{\prime t_2}_{1,1}}{s^{\prime t_2}_{2,2}}}{1-\dfrac{s_{1,0}}{s_{2,0}}} = \frac{c^0_1 + c_a}{\left(\dfrac{x^{\prime t_2}_2}{x_0}\right)^{2\,s_{1,0}/s_{2,0}}}$$

where c_a is the added amount. If the observations are made when $x^{t_1}_2 = x^{\prime t_2}_2$ we get

$$c^0_1 = \frac{c_a\left[c^{t_1}_{1,1}\left(1-\dfrac{s^{t_1}_{1,1}}{s^{t_1}_{2,2}}\right)-1\right]}{c^{\prime t_2}_{1,1}\left(1-\dfrac{s^{\prime t_2}_{1,1}}{s^{\prime t_2}_{2,2}}\right)}. \qquad (13)$$

For more complex systems similar formulas may be worked out, but in practice their usefulness will be limited on account of the experimental errors. The above also holds for frontal analysis in the field of adsorption and was first deduced by the author in connection with similar problems. The concentration change due to the sector-shape is not present there, as cylindrical columns are used.

Applicability of formulas in practice

The usefulness of Eq. (9) depends on the fact that $f_{i,m}$ is the same for all the components ($i = 1, 2, \ldots, m$) and cancels out in the calculations. It may not be out of place to make brief mention of its applicability in practice. For proteins we usually have $f = 1/(1 + g(c_{1,m}, \ldots, c_{m,m}))$ or $f = 1 - g(c_{1,m}, \ldots, c_{m,m})$ and we can often write

$$g(c_{1,m}, \ldots, c_{m,m}) = \sum_{j=1}^{m} k_{i,j} c_{j,m} \quad (m = 1, 2, \ldots, n).$$

where $k_{i,j}$ refers to the effect of component j on the sedimentation constant $s_{i,m}$ of component i.

For a two-component system we have for example

$$f_{1,2} = \frac{1}{1 + k_{1,1} c_{1,2} + k_{1,2} c_{2,2}}$$

$$f_{2,2} = \frac{1}{1 + k_{2,1} c_{1,2} + k_{2,2} c_{2,2}}$$

where $f_{1,2}$ and $f_{2,2}$ refer to the sedimentation of component 1 and 2 resp. in a two-component system with concentrations $c_{1,2}$ and $c_{2,2}$ resp. For Eq. (9) to hold, i.e. $f_{1,2} = f_{2,2}$, we must have $k_{1,1} = k_{2,1}$ and $k_{1,2} = k_{2,2}$. This condition is normally fulfilled for molecules of nearly the same shape and molecular weight which also is the most common case where the changes in the concentration discussed above are of any importance. Even if the condition is only approximately fulfilled the errors might nevertheless be small enough to make it applicable.

Johnston and Ogston have used $f_{1,2} = f_{2,2} = 1 - k(c_{1,2} + c_{2,2})$ with $k_{1,1} = k_{1,2} = k_{2,1} = k_{2,2}$.

The Johnston-Ogston effect will generally become smaller and smaller with decreasing concentration as $s^t_{m,m} \rightarrow s^0_m$ and $s^t_{m+1,m+1} \rightarrow s^0_{m+1}$ in Eq. (9). An alternative method for determining the concentrations is then to make a number of runs on the same mixture at decreasing concentrations, Eriksson (102). This means, however, a considerable amount of experimental work and calculations. On the other hand, the $s_{i,0}$ values have to be known when using the above-mentioned equations. In either case the uncertainties of extrapolating the sedimentation constants to zero concentration cannot be avoided. At lower concentrations the boundaries also tend to be unstable. The method to be applied has to be chosen to fit each special case. For systems with a great many components, the experimental errors are too large to allow the use of the above formulas.

Practical details

The sedimentation studies were carried out in a Svedberg oil turbine ultracentrifuge (97). The sedimentation diagram was obtained by plotting Z, the scale line displacement, against x, the distance from the centre of rotation. The observed sedimentation constants were reduced to standard conditions at 20°C and will hereafter be denoted by s_{20}, and are given in Svedberg units. Details concerning the calculation of the molecular weight and the estimation of the molecular shape and hydration from the f/f_0 values are given both in the monograph by Svedberg and Pedersen and in a review by Edsall (112). The wavelength used in the ultracentrifuge was 5460 Å.

Chapter VI

Changes in the molecular state following irradiation

The possible reactions that a protein may undergo as a result of irradiation producing changes in the molecular state have been much discussed (4, 2), and possible mechanisms are mentioned in chapter IV. Knowledge has been accumulated both from irradiation of protein monolayers and protein solutions.

It seems reasonable to believe that the reactions observed when a protein is irradiated in the form of a monolayer are not necessarily the same as those which would occur in bulk solution. In a monolayer the molecule may be partly unfolded, is restricted in its movement, and has a fixed orientation. The reactions taking place in monolayers are nevertheless of great interest as they may serve as a guide in the interpretation of the reactions in solution.

Mitchell and Rideal irradiated several proteins, among them insulin and ovalbumin, as monolayers using λ 2537 Å (15). As a result of these experiments they suggest that the principal process is photochemical hydrolysis of the –CO–NH– linkages adjacent to the aromatic side chains, the aromatic acids or chromophore residues then passing into solution. Among more recent investigations that by Kaplan and Fraser (16) may be mentioned. These authors are of the opinion that a more complicated mechanism takes place. They used monolayers of ovalbumin and irradiated at λ 2540 Å. Their suggestion is that the protein is first unfolded and then that in this changed molecule the energy from the chromophores is transferred to adjacent labile bonds such as the hydrogen bond involving the –OH group in tyrosine for instance, resulting in further denaturation and unfolding of the molecule. After this, energy from the chromophores is finally transferred to the peptide bond and splitting into fragments occurs. These fragments may be large or small. The latter explanation seems to fit quite well with what reasonably might be expected.

For proteins irradiated in solution, evidence exists that splitting into smaller fragments occurs at least when oxygen is present. Becker and Szendrö (86) give very convincing data showing that dilute egg white solutions irradiated in oxygen finally show no trace of protein, only dialyzable products are present. Roberts (113) after irradiating bovine serum albumin in oxygen detected tryptophyl and tyrosyl groups in the filtrate when the protein had been precipitated with trichloroacetic acid.

It is generally believed that the splitting in these cases occurs in the peptide bond but absolute evidence of this does not exist. There is evidence that the peptide bond is more susceptible to splitting than the –S–S– bond (2).

Detailed information of possible subunits and the binding of these in the protein molecule in question is also desirable in this connection. The findings of Svedberg and Brohult (11) that hemocyanin splits into subunits is an example where a break in the peptide bond can hardly be responsible for the splitting (see also below). Here the weak forces or bonds acting between the dissociable subunits have been destroyed.

Equally or more important than the splitting is the formation of aggregates upon irradiation (9, 10, 13). There seems to exist both an aggregation reaction taking place immediately, probably due to molecules with a fairly short reactive "lifetime" and one that takes place as a result of irreversible changes in the surface. Oxygen has been found to partly inhibit aggregation.

From the combined evidence from experiments in monolayers and in bulk solution it seems thus reasonable to believe that for a molecule containing subunits between which only fairly weak forces act a splitting into these subunits would be the first reaction. For a molecule with no subunits and where no easy splitting occurs aggregation with other molecules may be favoured. If the aggregation is prevented for instance by the presence of oxygen, unfolding and finally splitting in the peptide chain may be found.

Of the two proteins used in the present work the first, *Helix pomatia* hemocyanin, has a very high molecular weight 9×10^6 and is easily dissociable into subunits, the other, bovine serum albumin, has a molecular weight about 7×10^4 and only one end-group has been found in the molecule.

It is thus to be expected that the two proteins should act differently upon irradiation and as already mentioned *Helix pomatia* hemocyanin has been found to split into subunits (11, 12) and bovine serum albumin to aggregate (9, 10, 13).

PART 1. *Helix pomatia* hemocyanin

Helix pomatia hemocyanin is the respiratory protein in the blood of the mollusc *Helix pomatia*. For detailed general information the reader is referred to a review by Dawson and Mallette (44). The ultraviolet spectrum and the amino acid composition have been treated in chapter I. The sedimentation and dissociation properties will be treated below, as well as other properties relevant for the irradiation experiment.

Svedberg and Brohult (11) found that undissociated hemocyanin splits into halves upon irradiation with the whole ultraviolet spectrum. That this was a primary photochemical reaction was shown by the fact that the same result was obtained at room temperature and at −180°C. Brohult (12) continued the investigation. He found then that the splitting followed a logarithmic curve at first; later, aggregation reactions appeared.

An advantage with hemocyanin is that it can be investigated more or less as it occurs in nature; no complicated extraction methods are necessary as the hemocyanin can be considered as the sole protein constituent after low molecular compounds have been removed by dialysis. Furthermore, on account of its oxygen

combining capacity, it has a specific property, in a way, comparable to enzyme and hormone activity and the effect of irradiation on this specific property can also be studied.

Before entering into the description of the irradiation experiments it is necessary to deal somewhat with unirradiated hemocyanin.

Unirradiated hemocyanin, earlier investigations

Sedimentation behaviour and dissociation reactions

Already in 1928 Svedberg and coworkers started to investigate these matters.

A detailed investigation of the pH-stability of a number of respiratory blood proteins, among them *Helix pomatia* hemocyanin, was carried out by I. B. Eriksson-Quensel and Svedberg in 1936 (114). Here the blood was simply diluted with buffer solution and the sedimentation diagrams had the same appearance as those from crystalline hemocyanin earlier used, showing that very little other protein material was present.

It was found that for pH ~ 1 the hemocyanin is inhomogeneous, for pH 3.6–4.6 two components with $s_{20} = 100$ and 60 are present, for pH 4.7–7.0 only one component with s_{20} 100 exists and between pH 7.4 and 8.1 there are two components with $s_{20} = 100$ and 60. Around 8.1–8.2 three components may be observed with $s_{20} = 100$, 60 and 19. Up to pH 9.3 there is only one component with $s_{20} = 19$, at higher pH values a component with a still lower s_{20} value, around 12, appears. The dissociation in the pH region 4–9 was found to be reversible. The buffers used were made up from different salts but only the effect of pH was studied.

It was later found by Brohult and Claesson (115) and by Flamée (116) that hemocyanin dissociates under the influence of salt. Brohult (12) then investigated in more detail both the pH dissociation and to a certain extent the salt dissociation. He redetermined the molecular weights of the different components from new sedimentation and diffusion data. The values are found in Table 4.

The first dissociation component seems to be a half molecule and the second is generally referred to as an eighth molecule although it may actually be a smaller unit.

The concentration dependence of the sedimentation constants was studied for all three components. He also found that the stability region of the undissociated molecule could be extended over a much wider pH range if the salt concentration was very low and that dissociation occurred even near the isoelectric point if the salt concentration was very much increased. From light scattering experiments

Table 4. Molecular constants of the hemocyanin molecule and its dissociation components according to Brohult (12).

$(s_{20})_0$	D_{20}	V_{20}	$M_{s,\,D}$	f/f_0	$M_{\text{equ.}}$
103.0	1.07	0.738	8.91×10^6	1.45	8.50×10^6
65.7	1.41	0.738	4.31×10^6	1.40	
19.7	1.77	0.738	1.03×10^6	1.79	

Brosteaux (117) had earlier demonstrated that hemocyanin shows a specially great stability in the presence of Ca– and Mg–ions. Dissociation does then not occur until a pH above 9.

Products intermediate between the undissociated molecules and the first dissociation component are often found in the ultracentrifuge diagrams. Brohult assumes them to be transition forms between the two molecules, that is undissociated molecules that have swollen and in this way changed their form with a lowered sedimentation constant as a result. That the intermediate products did not consist of wholes formed during centrifugation was proved by Brohult (12, 118).

Heterogeneity

The opinion that hemocyanin might not consist of only one kind of molecules has been ventured by several workers. Roche and Roche (119) found evidence of different kinds of hemocyanin molecules in the same preparation and suggested that slow aging processes in the blood and the time of the year when the blood was taken played an important role. As a result of the reversible boundary spreading found in electrophoresis, Tiselius and Horsfall point out the possible existence of different kinds of molecules with very nearly the same molecular weight (49). Linnman (120), when making a sedimentation study of hemocyanin from a number of individual snails at a pH close to the edge of the stability region for the undissociated molecule, found marked differences in dissociation behaviour not only between the hemocyanin of the different snails but also in the hemocyanin of the same snails from which blood was taken at different times.

As hemocyanin could not be dissociated more than to 75 per cent in concentrated sodium chloride solutions, Brohult and Borgman (121) were inclined to believe in the existence of at least two different kinds of hemocyanin molecules. Brohult (118) was also able to separate them by fractional precipitation with ammonium sulfate and also by preparative centrifugation and showed that the components differed in their behaviour to sodium chloride.

Lontie and coworkers have investigated the influence of salt and pH on the dissociation of hemocyanin (122) and found, mainly from light scattering measurements, that the so called β-component (not dissociating in 1 molar sodium chloride) also had a wider pH-stability region towards the alkaline side. The experiments indicate that it was broken down immediately to the second dissociation component without the first showing up. For the β-component a molecular weight somewhat higher than for the α-component was found by Borginon (cited in (122)) from light scattering measurements. Both were $7.5—8 \times 10^6$. These values are lower than that mentioned above from sedimentation and diffusion, 9×10^6.

Separation according to the behavior towards strong sodium chloride is only one of the ways of separating the hemocyanin into fractions. Recent chromatographic work by Tiselius (17) shows another possible way.

Electron microscopy

The form of the hemocyanin molecule and its dissociation components have been under much discussion. In the electron micrograph study by Schramm and Berger (123) the different views prevailing up till then are recorded. These authors found that the undissociated molecule had a length of about 400 Å and that it consisted

of two cylindrical subunits in which further structure could be seen. The diameter of the subunits is given as 120 Å and the resulting molecular weight around 7×10^6. The first dissociation component would thus be one of these cylinders. The second dissociation component was reported to have an almost sphere-like shape with a diameter around 100 Å. The f/f_0 values from the sedimentation and diffusion data given by Brohult indicate a very unsymmetrical form for the second dissociation component and it is difficult to reconcile this fact with a sphere-like form.

Unirradiated hemocyanin, present investigation

The present author made a number of ultracentrifugations on unirradiated hemocyanin solutions for comparison with the irradiated ones.

Preparation and examination of the material

It is obvious that when working with a hemocyanin preparation resulting from 50-100 snails a certain heterogeneity in the material has to be expected and that one preparation may differ somewhat from another.

The hemocyanin was mostly prepared as described by Brohult (12). The whole blood taken from the snail by heart puncture was allowed to stand for a day or two at +4°C and was then centrifuged at 3500 r.p.m. to get rid of a precipitation sometimes found. The concentration of this hemocyanin solution was 2–3 % for summer snails and around 5 % for the hibernating ones. A concentration of up to 0.7 % salt was also observed. The salt composition of the blood has been determined by Lustig, Ernst and Reuss (124). In some cases this salt was entirely removed by dialysis against distilled water.

Hemocyanin precipitated by ammonium sulfate near the isoelectric point and then redissolved was used in a few cases. The preparation procedure was similar to that described by Lontie *et al.* (45). In the ultracentrifuge the appearance of these solutions was the same as for diluted blood.

The ultracentrifuge diagrams of preparations consisting of diluted blood were studied at the highest scale distance both in front of and after the hemocyanin peak, at a speed of 24,000 r.p.m. and 64,000 r.p.m. resp. The statement already given by Brohult (12) that at least about 95 % of the material sediments in the hemocyanin peak was confirmed. The possibility that lower products are absorbed onto the surface of the hemocyanin can of course not be excluded. Chromatographic methods might give the answer to this.

Part of the concentrated stock solution was kept at +4°C for use during a few weeks, the rest was immediately frozen down in small portions (5–10 ml) and kept at −10 C. After thawing, all the hemocyanin of such a portion was used up during a short time; no refreezing was ever done. Freezing, though mostly without effect on the sedimentation behaviour, has been found to lead to dissociation at times. Every portion was tested in the ultracentrifuge before further experiments were carried out. Only those solutions showing no dissociation under the chosen standard conditions, phosphate buffer pH 6.2, containing 0.1*M* sodium chloride, were used.

Tiselius and Horsfall (49) found that the electrophoretic properties of hemocyanin changed when the solution was kept at +4°C but that they remained the same if the solution was stored frozen at −10°C.

In the present investigation it was found that solutions stored frozen in some cases showed greater tendency to split upon irradiation than those stored at +4°C. When possible, storage at +4°C was therefore used.

The use of toluene as a preservative did not affect the appearance of the ultracentrifuge diagram either for frozen or unfrozen preparations. However, in most cases storage at high concentration without toluene was used.

Freeze drying denatures the hemocyanin whether it is frozen in distilled water or in buffer solution. In the presence of sugar freeze drying has proved successful, however (Lontie, private communication). Denatured, freeze dried material was used in a few ultracentrifuge runs in order to get diagrams from hemocyanin changed by other means than irradiation for purposes of comparison.

Investigations were also performed with material where the α- and β-components were partly separated by preparative centrifugation in a medium (1*M* NaCl in acetate buffer of pH 5.3) where the α-component is completely dissociated and the β-component unaffected.

Hemocyanin fractions obtained by chromatographic methods using frontal analysis with active carbon as adsorbent were also used. The frontal analysis curves showed that the substance contained a number of components not differing much in adsorption. When examined in the ultracentrifuge the different fractions had the same sedimentation constant and furthermore, the ratio between the optical density at 3460 Å (the copper band) and at 2780 Å (the protein band) was constant for the fractions and had not changed from that of the original solution. In consequence very little other protein material than hemocyanin is likely to be present in agreement with the measurements from ultracentrifugation. If the intactness of the copper band can be taken as a proof of intactness of the whole molecule, no denaturation has occurred. It appeared that for undissociated molecules the first fraction had a higher percentage of the β-component (stable against NaCl) than the original solution but the difference in adsorption between the two components was so small that preparation of pure β-component in this way was not practical. An interesting thing was that the same amount of material is adsorbed by the carbon from a solution of undissociated molecules at pH 6.2 as from one containing only the second dissociation component at pH 8.2. However, closer investigation is necessary to determine whether this means that the adsorption process is the same in both cases. The work by Tiselius (17) in this connection has already been mentioned.

Determination of concentration

The concentrations of the hemocyanin solutions were determined refractometrically at a fairly high concentration (1–2 %) using the value 186.3×10^{-5} for the refractive index increment at 5460 Å (49). The refractive index is not dependent on the dissociated state. This solution was then diluted to the desired concentration (0.1–1 %). The use of refractive index for determining concentrations was chosen as being the most practical since all concentration determinations in the ultracentrifuge make use of it. The optical density at 2780 Å gave reliable values for the concentration in the cases where either only undissociated molecules were present or only the second dissociation component (see chapter I in connection with the spectrum). If the salt had been removed from the stock solutions the concentration was also checked by weighing and drying at 105°C. The nitrogen content was deter-

mined for some preparations and was found to be around 15.2 %. Values around 15.15 % are usually mentioned in the literature (12, 45, 50) but some as high as 15.43 % have been reported (49). The amino acid composition has already been given in chapter I.

Electron microscopy

The present author made routine investigations of some of the hemocyanin preparations in the electron microscope. The instrument was a Siemens ÜM 100 and had rather low resolution, about 50 Å. The magnification was around 8500. Gold shadowing was used in all cases with angles varying from 1: 2 to 1: 6. Although in some cases the solution contained salt the structure was still fairly clearly seen. Schramm and Berger used salt free preparations that had been treated with osmium oxide and claim that the structure is almost wiped out otherwise.

From the investigations the following can be reported. In all cases where solutions of undissociated molecules were used square-like forms appear (Fig. 26), each of which exhibits a definite structure in agreement with the pictures by Schramm and Berger (123). Shadowing at low angles (1: 6) shows a rather wedgelike form of the shadow. It is, however, difficult to say if the molecules are ellipsoidal or cylindrical. The length is somewhat larger, average 425 Å, than reported by the previous workers. The width of the molecule is also larger, about 350 Å, giving 175 Å for each subunit. For the second dissociation component it is difficult to give any exact data (Fig. 26, *4*). An average value of about 100 Å is, however, indicated. Whether the high f/f_0 value (see Table 4) for this component depends on an extremely uneven surface (123) or if the molecule has a more uncoiled form in solution than when dried on the electron micrograph is impossible to say.

The evidence from the electron microscope thus indicates that hemocyanin has at most a length of 450 Å. No association of molecules end to end is found. A length of around 1000 Å (12, 118) in solution would necessitate that a folding always occurred on drying.

Conditions for ultracentrifugation

It has already been mentioned in chapter V that the detailed description of the ultracentrifuge used is to be found in Svedberg and Pedersen's monograph (97) and that Lamm's scale method was used. In chapter V the formulas etc. are also given.

Undissociated hemocyanin has a high sedimentation constant and a low diffusion constant, see Table 4, the peaks in the ultracentrifuge diagram will then be very sharp and the concentration determination from the areas rather difficult. In a cell 12 mm thick the highest concentration that can be determined with any accuracy is 0.2 %. Scale distances that are higher than 20 mm cannot be used here. For a cell thickness of 4 mm the scale distances 40 60 mm can be used, however, and for undissociated hemocyanin such a cell is then to be preferred. Equilibrium centrifugations are suited for the study at higher concentrations but are rather time consuming. When running mixtures of the different hemocyanin components it is, however, important to be able to detect small amounts of both the first and the second dissociation component. A cell thickness of 12 mm is then better suited for this problem than one of 4 mm.

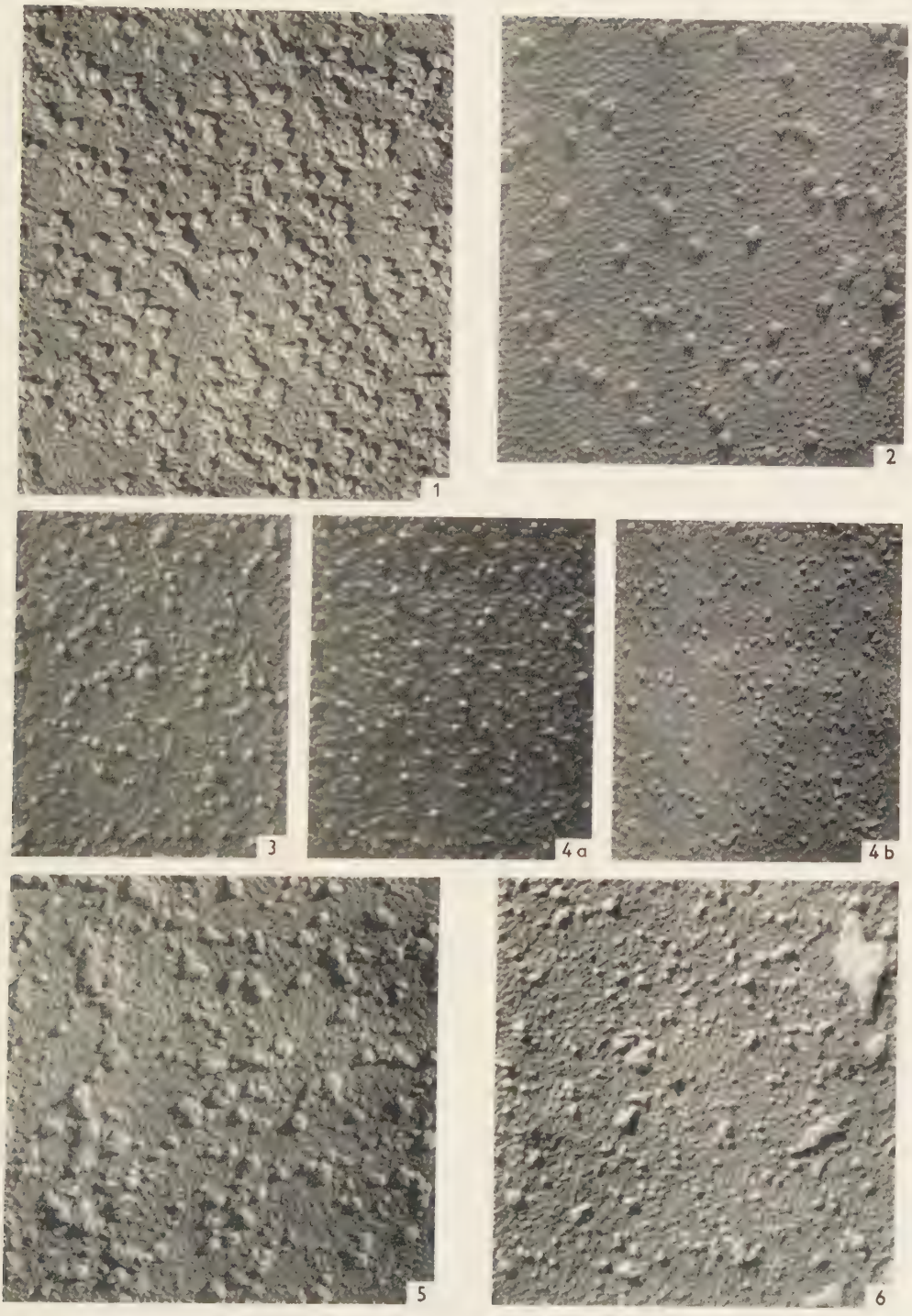


Fig. 26. Electron micrograph of hemocyanin from dried dilute solutions. **1** In water, **2** in phosphate buffer pH 6.2, **3** in phosphate buffer pH 7.6, **4a** and **b** in borate buffer pH 8.2, **5** and **6** irradiated with $Q/d_{2537} = 3 \times 10^{18}$ $h\nu$/ml in phosphate buffer at pH 6.2 and pH 7.6 resp. The magnification is about 43,000 times.

The speed used was mostly 24,000 r.p.m., (about 45,000 *g*) both for runs with the undissociated form alone and when in mixtures of the other components. The sedimenting boundary of the undissociated form then reached the bottom of the cell in about 50 minutes. When the second dissociation component has been especially studied the speed has been 36,000 r.p.m. The concentration has decreased to 0.7 c^0 when the boundary is close to the bottom of the cell (see chapter V).

Sedimentation behaviour

Ultracentrifugations of the unirradiated hemocyanin were performed at different concentrations principally at pH 5.3, 6.2, 7.6 and 8.2 in buffer solution and in distilled water with a pH around 7; these pH values were also used by Brohult (12). In all cases except for those run in distilled water enough salt was present to eliminate the Donnan effect.

The composition of the buffers was 0.08*M* acetate pH 5.3 + (0.1 − 1)*M* NaCl 0.08*M* phosphate pH 6.2 and 7.6 + (0.1 − 1)*M* NaCl and 0.03*M* borate-phosphate pH 8.2 + (0.1 − 1)*M* NaCl. Typical diagrams are shown in Figs. 27 and 28. The phosphate buffer pH 6.2 containing 1.0*M* NaCl has a pH around 5.9.

The peaks in the ultracentrifuge diagrams that represent the undissociated molecules at pH 5.3 and 6.2 are, as already mentioned, extremely sharp even at low concentrations (0.1–0.2 %). A slight broadening, see Fig. 27, of the peaks occurs, however, and must then either be due to slight polydispersity of the material or to instability of the sedimenting boundary. The broadening is proportionally larger at the base of the peak than at the top. Gralén (125) in his work on cellulose encountered similar but more pronounced effects due to polydispersity. When calculating the areas of the peaks he drew the baseline in the diagram so as to make the areas fit with the actual concentrations. This is also the only possibility for the sharp peaks of the undissociated hemocyanin. On both sides of the peak the base line has to be examined at the highest scale distance to make certain that no other

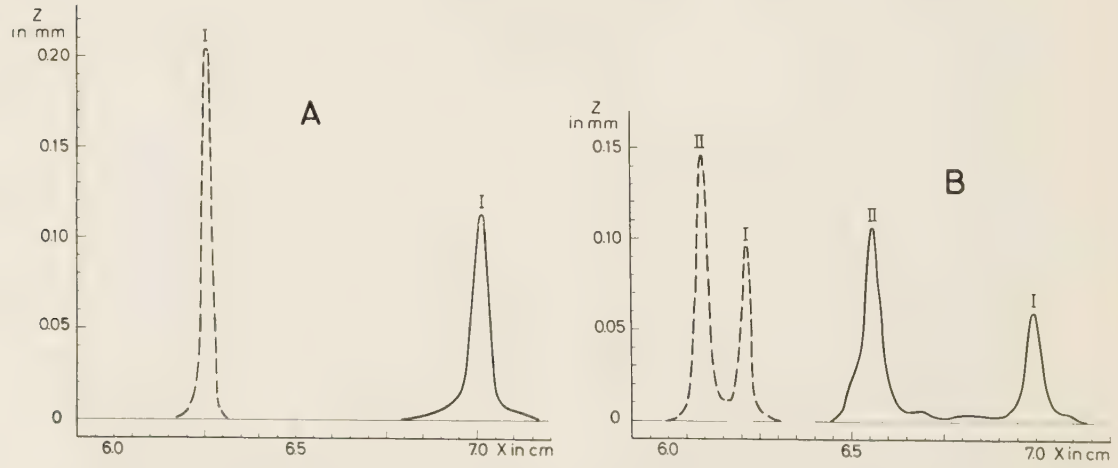


Fig. 27. Ultracentrifuge diagrams of unirradiated hemocyanin. **A** 0.2 % at pH 6.2 with 0.1*M* NaCl, **B** 0.3 % at pH 5.3 with 1*M* NaCl. Time and scale distance **A** 10^m and 40^m, 20 mm, **B** 10^m and 50^m, 20 mm. 24,000 r.p.m. I marks undissociated molecules, wholes, II marks the first dissociation component, halves.

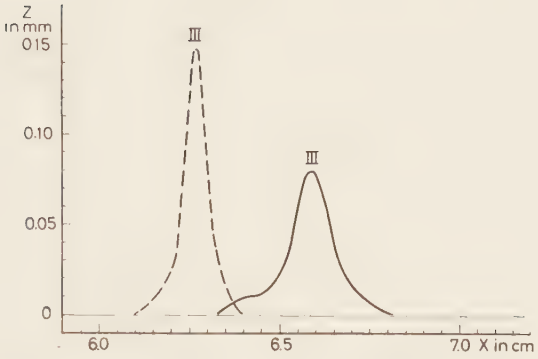


Fig. 28. Ultracentrifuge diagram of unirradiated hemocyanin. 0.2 % at pH 8.2 with 0.1*M* NaCl. Time and scale distance 40[m] and 80[m], 40 mm. 36,000 r.p.m. III marks the second dissociation component, eighths.

hemocyanin components are present. The base lines drawn in this way are generally in agreement with the experimental points of the curve, but have a broader base of the peak than that of an ideal symmetrical one, which accordingly gives an area which is too low. The errors in the area determinations are at least 5 %. The broadening of the peaks is more marked at low concentrations, consistent with the view that the Johnston-Ogston effect is lower here (see page 55). The sedimenting boundary will generally tend to be more unstable at low concentrations but for these sharp peaks it seems to keep well together at least down to concentrations of 0.02–0.03 %.

Figs. 29 and 30 show the concentration dependence of the sedimentation constant, it is seen that it is more marked at pH 6.2 than at 5.3.

The extrapolated values at zero concentration $(s_{20})_0$ for the two curves do not seem to differ much, however; nor is the diffusion constant markedly lower at pH 6.2 as would be expected for an increased frictional resistance. The diffusion constants were determined in a new diffusion set-up at $c = 0.3$ %. The values at 20°C were $D_A =$

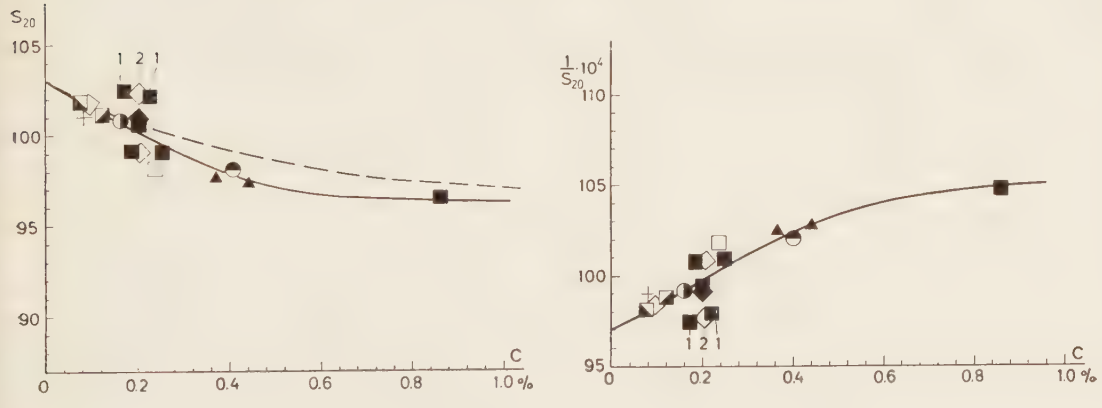


Fig. 29A and B. Concentration dependence of sedimentation constant for whole molecules. The different points represent various preparations in acetate buffer at pH 5.3 containing 0.2*M* NaCl for ■ and □, 0.1*M* NaCl for the rest. Dashed curve according to Brohult (12). Solutions at 1 and 2 have been frozen.

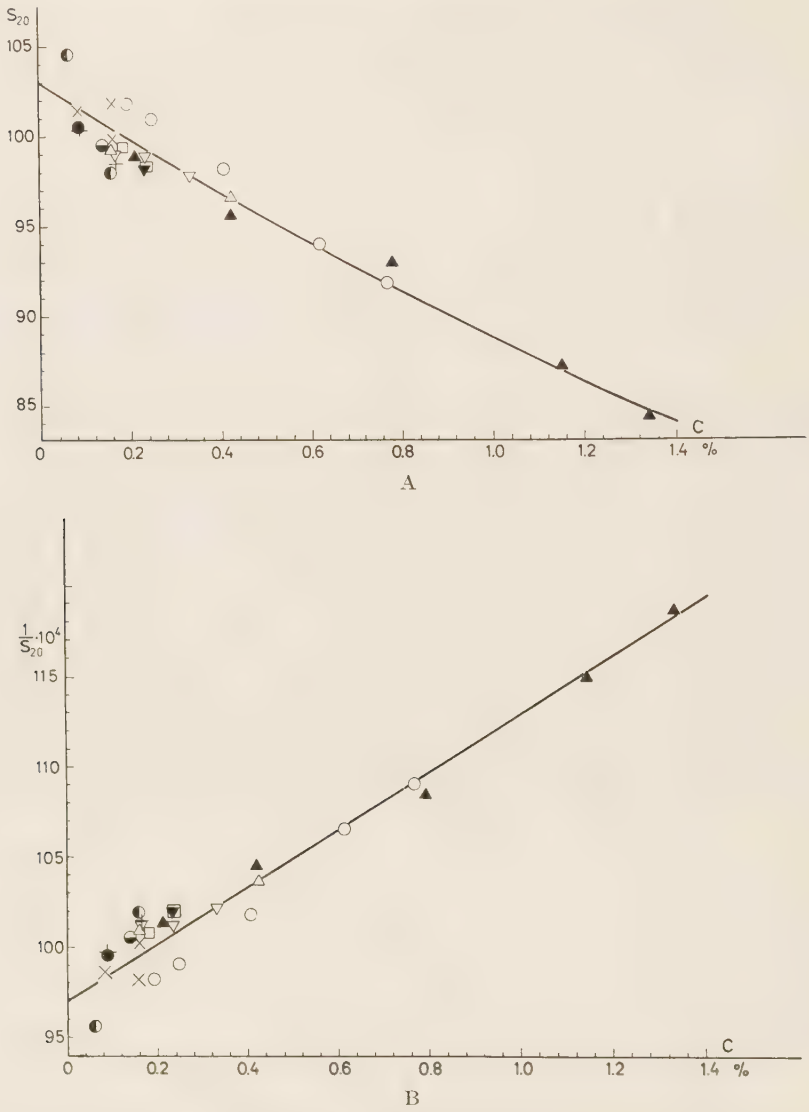


Fig. 30 A and B. Concentration dependence of sedimentation constant for whole molecules. The different points represent various preparations in phosphate buffer at pH 6.2 containing 0.2*M* NaCl for ○ and ◐, 0.1*M* NaCl for the rest.

$= 1.05 \times 10^{-7}$ at pH 5.3 and $D_A = 1.03 \times 10^{-5}$ at pH 6.2 The author is indebted to Mr. L.-O. Sundelöf for assistance with these measurements. The values are in good agreement with those published earlier (12). As to whether the extrapolated $(s_{20})_0$ value is correct and if it can be used together with a diffusion constant measured at 0.3 % for the molecular weight determinations is of course not certain. It seems, however, reasonable to believe that the value $(s_{20})_0 = 103$ is fairly correct since, together with

the diffusion constant $D_{20} = 1.07 \times 10^{-7}$, which is not expected to change very much with concentration (determinations at very low concentrations are still not available) Brohult got about the same molecular weight as that from equilibrium centrifugations. The light scattering measurements by Borginon cited in ref. (122) give, as already mentioned, $(7–8) \times 10^6$.

The light scattering measurements mentioned above gave a slightly higher molecular weight for the β-component and the possibility that the sedimentation constant also might be higher was somewhat investigated.

Fractions from solutions run in the Spinco centrifuge in the previously mentioned acetate and phosphate buffers with 1M NaCl were run in the ultracentrifuge. Three fractions were investigated and they contained 5, 20 and 50 % β-component respectively. The sedimentation constants of the β-component in this strong salt medium (Fig. 35) generally show a rather large variation. Values as high as 110–115 have been obtained from some preparations whereas values around 100 are obtained in other cases. These irregularities are probably the effect of the strong salt medium and make it impossible to draw any definite conclusions. The fractions were dialyzed against acetate and phosphate buffers at pH 5.3 and 6.2 containing 0.1M NaCl and then run in the ultracentrifuge at concentrations 0.1–0.2 %. Complete reassociation was obtained and the variation in the appearance of the peaks was not larger between the fractions than between two runs on identical solutions. There was no special broadening in front of the peak in the solution containing high percentages of β-component. Thus there does not seem to be any reason for assuming a markedly higher sedimentation constant for the β-component than for the α-component. It is true that strong displacement occurs for molecules with nearly the same sedimentation constant, see Fig. 25, but for $s_{1,0}/s_{2,0} < 0.90$ a displacement of up to 50 % seems improbable.

In this connection experiments were also undertaken to find out if the β-component increased in the blood when it had been kept for some time in the refrigerator. The β-component could for instance be an aged molecule and perhaps the first stage in the destruction of the hemocyanin molecule, compare Roche and Roche (119), page 59, above. However, no such change could be detected in the amount of the β-component measured in strong sodium chloride solution. This does not exclude the possibility that the β-component could be a transformed α-component or vice

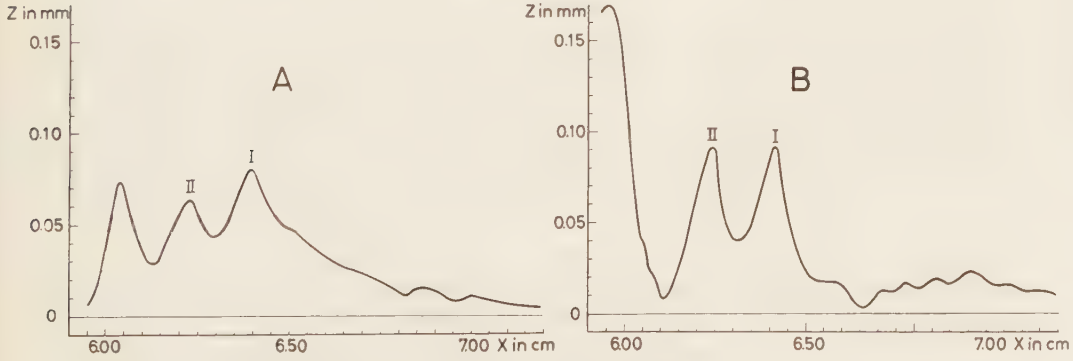


Fig. 31. Ultracentrifuge diagram of freeze dried hemocyanin. **A** 0.5 % at pH 5.3, **B** 0.3 % at pH 6.2, both with 0.1M NaCl. Time and scale distance **A** 15^m, 120 mm, **B** 20^m, 120 mm.

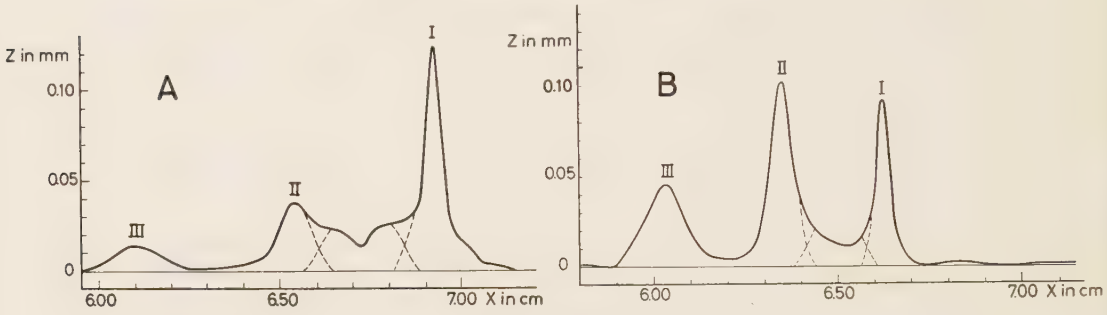


Fig. 32 A and B. Ultracentrifuge diagrams of unirradiated hemocyanin, **A** 0.10 % at pH 7.2 and **B** 0.13 % at pH 7.6, both with 0.1*M* NaCl. Time and scale distance 40^{m}, 120 mm for both.

versa—it only means that the conditions used did not promote such a transformation. In ref. (12) it is mentioned that Claesson obtained an electro-dialyzed hemocyanin preparation that did not dissociate in strong sodium chloride. It will also be shown later, page 80, that irradiation produces such molecules.

Some preparations from hibernating snails were found to contain a much lower percentage, down to 5–6 %, of the β-component than is usually found, 15–25 % Linnman (120) and 25–30 %, Brohult (118), Lontie (122); preparations from summer snails always had a higher percentage, 20–25 %. The observed differences may or may not be significant but are primarily of biological interest.

It is interesting to compare the ultracentrifugation diagram of material destroyed by freeze drying, see Fig. 31. Great polydispersity is indicated in both cases, but at pH 5.3 more aggregates with higher sedimentation constants are seen whereas products with lower sedimentation constants are more abundant at pH 6.2.

At pH 7.6, undissociated molecules (wholes) and the molecules of the first dissociation component (halves) normally appear. In some frozen preparations the second dissociation component (eighths) is also seen. Fig. 32 shows such a preparation at pH 7.2 and pH 7.6. At the lower pH 7.2 the dissociation is less marked. The peak of the first dissociation components is not quite as sharp as that of the undissociated material, the diffusion constant is also higher, 1.41 Table 4.

Between the two main components a certain amount of intermediate components is observed. That these are not recombined molecules formed during the centrifugation has as already been mentioned been proved by Brohult (12). His later work (118) also indicates that all the different stages of dissociation from mere loosening of the structure to the complete separation of the two subunits are represented.

The concentration dependence of the sedimentation constant for the first dissociation component was studied at pH 7.6 where it sediments in a medium made up only of its own molecules, Fig. 33. The sedimentation behaviour of the undissociated molecules which sediment in a "mixed medium" in this case will be treated later.

At salt concentrations as high as 1*M* NaCl in the buffers of pH 5.3 and 6.2 the first dissociation component also appears, as has already been mentioned. The data from these measurements are also found in Fig. 33.

For the second dissociation component studied at pH 8.2 where it appears alone the peaks are broad enough to enable calculation of the areas to be made without difficulty. A slight tail of the peak in certain preparations indicates the presence of

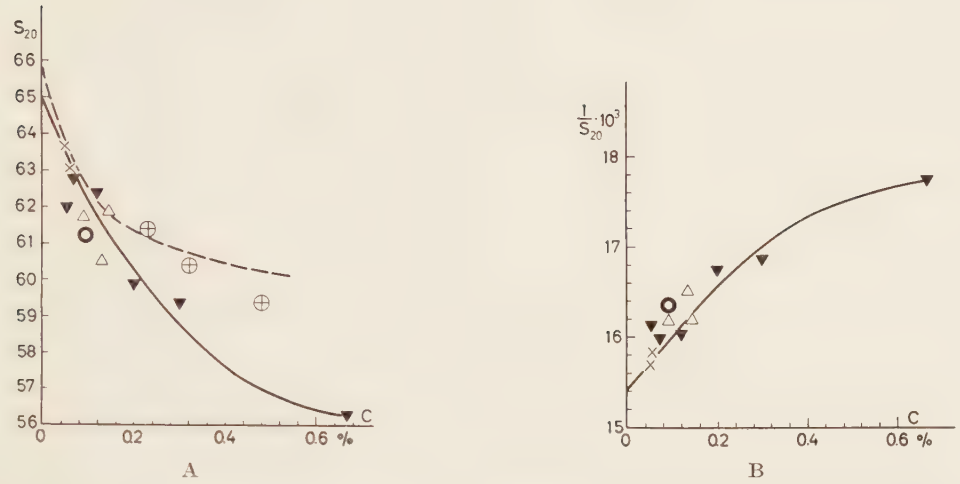


Fig. 33A and B. Concentration dependence of sedimentation constant for half molecules. The different points represent different preparations in phosphate buffer at pH 7.6 with 0.2*M* NaCl, except ⊕ which are at pH 5.9 with 1.0*M* NaCl. Dashed curve according to Brohult (12).

molecules with lower sedimentation constants $s_{20} \sim 12$. This more slowly moving component normally shows up at pH higher than 9 (114).

On standing, the amount of the slow moving component increases slowly showing that the second dissociation component is not very stable in itself. The electrophoresis pattern is also known to change (12). On increasing the salt concentration to 1*M* NaCl (pH = 7.8) no increase of the slow moving component was observed. Whether salt splitting of the second dissociation component occurs at higher pH's or at higher salt concentrations has not been investigated. The concentration deendence of the sedimentation constant is seen in Fig. 34.

The dissociation of hemocyanin seems to be completely reversible as long as the second dissociation component has not appeared. However, when this has happened the recombination is not reversible possibly because subunits of the β-component

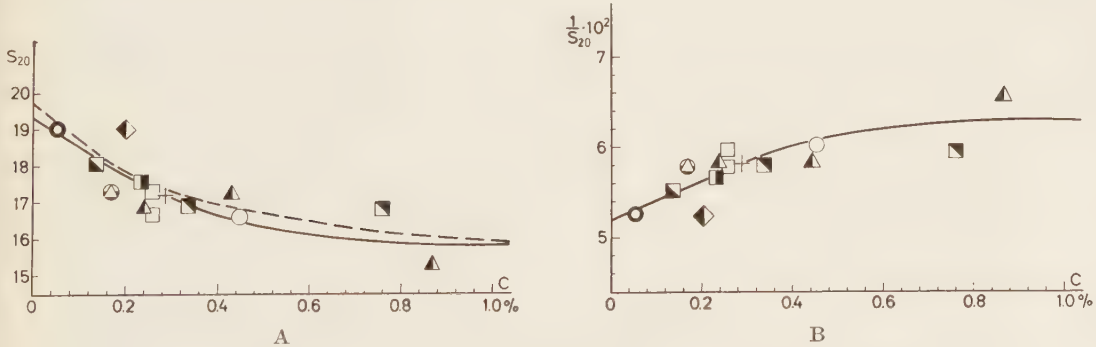


Fig. 34A and B. Concentration dependence of sedimentation constant for eighth molecules. The different points represent various preparations in borate buffer at pH 8.2 containing 0.1*M* NaCl for ◒ △ □ and +, 0.2*M* NaCl for the rest. Dashed curve according to Brohult (12).

Table 5. Values of k for c in % from the expression $s = s_0/(1 + kc)$.

Substance	pH	k
Hemocyanin wholes	5.3	0.13
wholes	6.2	0.16
halves	7.6	0.38
eighths	8.2	0.37

and the α-component do not readily combine. It may be mentioned in this connection that Tiselius and Horsfall (126) report that no component other than whole molecules was to be found after dialysis from pH 8.5, where only the second dissociation component was present, to pH 6.8. They used a concentration of about 0.4 %. The sedimentation constant for the reassociated material was considerably higher, 98.6 than that originally, 92.7 at pH 6.8. It is thus possible that some change had occurred after all as dilution at dialysis cannot account for such a large change.

If a hemocyanin solution is left for some time at pH 8.2 where only the second dissociation component is present the recombination to undissociated molecules at pH 5.3 is even less complete than if an immediate change in pH is brought about. The special surface structure necessary for recombination is more or less destroyed. A statistical reasoning at once reveals that even a low percentage of changed or different subunits must interfere drastically with recombination of so many units.

A few ultracentrifugations were performed on solutions at pH 3–4 and dissociation products were found in agreement with earlier investigations. The diagrams are however less well defined in this region, and pH's acid to the isoelectric point were therefore not used in this study.

The component with $s_{20} \sim 12$ was not investigated closer but diffusion data from mixtures of this component and the eighths give evidence that it has a lower molecular weight than the eighths.

The concentration dependence of the sedimentation constants of the different components and the sedimentation in media containing several components will now be treated in greater detail. The theoretical treatment is found in chapter V. To every calculated s-value the concentration corresponding to the x^m value is taken.

The expression $1/s = (1/s^0)\ (1 + kc)$ does not seem to represent the concentration dependence very adequately, Figs. 29, 30, 33 and 34. If, however, the best possible line is drawn and k calculated, the values in Table 5 are obtained.

It is specially noticeable that the concentration dependence is strong for the halves and the eighths. Brohult took this as evidence that the eighths were very unsymmetrical in form. Since the f/f_0 values point in the same direction one is again inclined to question whether the electron micrographs show the molecular form present in solution.

When the undissociated component is present together with the first dissociation component at pH 7.6, the sedimentation constant does not seem to change markedly for the former, see Fig. 35. From the k-values in Table 5 a greater change would be expected.

No signs that point at a difference in the sedimentation constant between the α- and β-components of the undissociated material are seen either at pH 7.4 or at

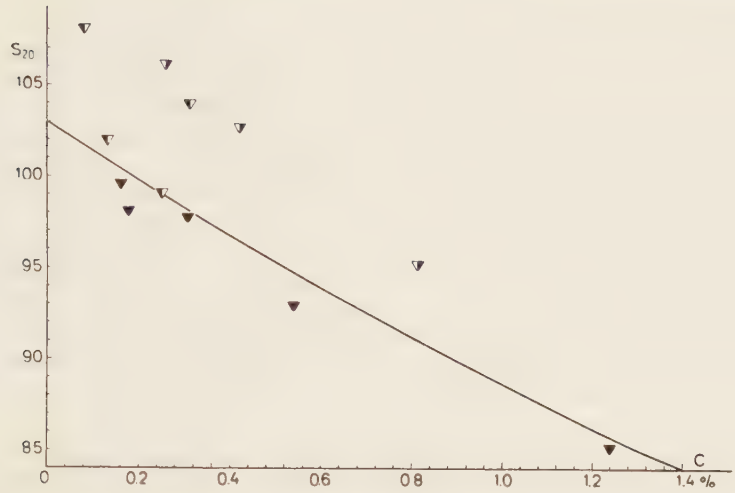


Fig. 35. Concentration dependence of sedimentation constant for whole molecules in the presence of half molecules, c is total concentration, ▼ pH 7.6 with 0.2*M* NaCl, ▼ pH 5.3 with 1*M* NaCl, ▼ pH 5.9 with 1*M* NaCl. The curve is the same as in Fig. 30A.

pH 7.6. The β-component remains, according to Lontie (122,) undissociated at this pH.

Just after the occurrence of dissociation the sedimentation constant of the undissociated material varies in a peculiar way. In 1 molar sodium chloride at pH around 6 Brohult observed that the sedimentation constant increases up to around 120 in phosphate buffer and to around 110 in acetate buffer whereas in unbuffered sodium chloride at pH 6.9 there was no increase. At higher salt concentrations the sedimentation constant again became lower. Brohult suggested that some aggregation reaction was responsible. When the dissociation is brought about by a change in pH a similar increase is found in the sedimentation constants as reported by I. B. Eriksson-Quensel and Svedberg (114). Experimental errors cannot explain the general trend observed there both for *Helix pomatia* hemocyanin and for other hemocyanins. The values given in the last mentioned paper also show that a slight change in sedimentation constant occurs at pH's where no dissociation is found if the buffer system is changed.

There thus exists evidence that the sedimentation constant of the component that starts to sediment in a medium also containing dissociation products instead of in a medium of its own molecules, increases. As the sedimentation constants often are higher than the values from extrapolation to zero concentration inside the stability region, a change in the molecule itself is the probable reason. The tempting explanation that a considerably higher sedimentation constant of the β-component should be responsible for the effect has to be rejected as a large proportion of the undissociated molecules immediately after dissociation still is the α-component.

Just before dissociation occurs a decrease in the sedimentation constant is observed. For a concentration around 0.2 % a value as low as 89 was observed by the above workers (114). Such changes certainly must be due to changes in the molecular shape preceding the dissociation.

From the theory developed in chapter V and the k-values given in Table 5 it is easily found that the Johnston-Ogston effect is small for hemocyanin solutions containing both whole molecules and their dissociation components at the total concentrations normally used, 0.2 %. See also Fig. 25 where curves for the ratios $s_{1.0}/s_{2.0}$ valid for the different hemocyanin components are found.

The displacement effect was also studied experimentally by ultracentrifugation of a hemocyanin solution at pH 7.58 containing whole and half molecules at different total concentrations. 0.35. 0.18 and 0.085 %. In these cases the displacement was found to be within the limits of the experimental errors in the concentration determinations. However, at concentrations as high as 0.7 and 1.4 % displacement effects > 5 % could be observed. Here only the sedimentation constants and the concentrations of the halves were determined. The peaks were too sharp for a determination of the concentrations of the wholes.

Stability region

The pH-stability region of hemocyanin in the buffer systems mostly used in the irradiated experiments will now be treated.

In the investigation by I.-B. Eriksson-Quensel and Svedberg (114) of the pH-dependence of the dissociation of the hemocyanin molecule the experiments were performed in 0.08M acetate buffers for pH 3.6–5.4, in 0.08M phosphate buffers for pH 5.5–8.0, and in 0.03M borate buffers for pH 8.1–9.3. 0.1M sodium chloride was in each case added to eliminate the Donnan effect in the ultracentrifuge. The effects of different salts observed by other workers (see page 58) made it probable, however, that the stability regions of the different hemocyanin components might change with the buffer used. Instead of using a large number of ultracentrifugations for the investigation of this matter the present author decided to make use of the difference in the light scattering contribution in the ultraviolet spectrum of the different components, see chapter I. Dissociation will thus be shown by a decrease in the extinction coefficient. Lontie *et al.* (45) used this method when studying the pH-stability of hemocyanin.

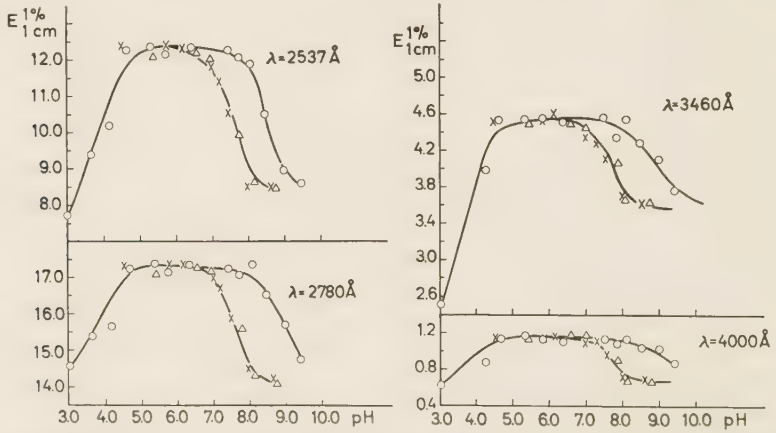


Fig. 36. Stability diagrams for hemocyanin as obtained from extinction measurements at four different wavelengths, ○ acetate medium, × phosphate medium, △ borate–phosphate medium. 0.1M NaCl in all cases.

The extinction coefficients at 2537 Å, 2780 Å, 3460 Å and 4000 Å were determined from pH 3.0 to 9.0 at intervals of about 0.1 in the pH value, Fig. 36. In one series of experiments the medium was 0.08*M* in acetate and 0.1*M* in sodium chloride for another 0.08*M* in phosphate and 0.1*M* in sodium chloride and in still another series of experiments in a medium of 0.03*M* phosphate–borate and 0.1*M* sodium chloride. The buffer capacity is of course very low at certain of these pH values but quite sufficient to keep the pH constant during an ultracentrifugation or a measurement of the absorption spectrum. All pH values between 3–9 cannot be realized with the same buffer system. From the curves in Fig. 36 it is seen that in the acetate medium the undissociated form is present in a wider pH region than in the other buffer systems.

The experimental error when measuring the extinction coefficient is not small enough to make it possible to decide if dissociation sets in to a very small extent already inside the "stability region". At lower salt concentrations, total absence of sodium chloride for instance, the stability region is somewhat increased but even in 0.015*M* borate–phosphate buffer at pH 8.2 only eighths are found. Ultracentrifugations were performed on a few of the solutions and in all cases confirmed the results from the extinction measurements.

On the whole it is clear that the stability of the undissociated molecule is extremely dependent on both the pH and the individual salts present; the molecule is actually a different one from case to case. This has to be kept in mind especially when comparing effects of irradiation.

Irradiated hemocyanin

The solutions of irradiated hemocyanin present a rather complicated picture. The resulting reaction is highly dependent on the composition of the buffer system, as is to be expected from the above mentioned description of the stability diagram.

The experimental arrangements at irradiation were those described in chapter II.

Investigations were first made to determine whether the irradiated solutions were stable long enough after irradiation to make ultracentrifuge analysis possible. Several experiments were performed where samples of the same solution were run in the ultracentrifuge immediately after irradiation and at different times, up to one week after irradiation. It is seen in Figs. 37, 38 and 39 that during this time the ultracentrifuge diagram does not change noticeably even in the case where aggregation has occurred and where a decreased stability might have been expected. The possibility of reactions taking place at such a high rate immediately after irradiation that they already are over after a few minutes can of course not be

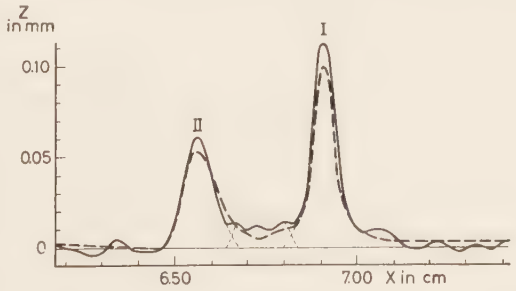


Fig. 37. Ultracentrifuge diagram of hemocyanin 0.1 % at pH 6.2 containing 0.1*M* NaCl, irradiated in air with $Q/d_{2537} = 5 \times 10^{18}$ $h\nu$/ml. Solid line obtained directly after irradiation, dashed line one week later. Time and scale distance 40^{m}, 120 mm.

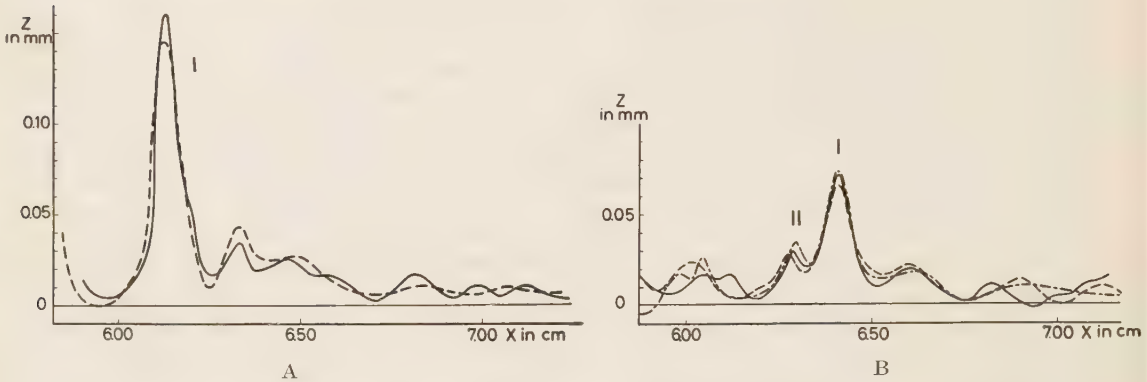


Fig. 38A and B. Ultracentrifuge diagram of hemocyanin 0.1 % at pH 5.3 containing 0.1*M* NaCl, irradiated in nitrogen with $Q/d_{2537} = 3.4 \times 10^{18}$ $h\nu$/ml. Solid line obtained directly after irradiation, – – – 3 hours later, and –·– 24 hours later. Time and scale distance **A** 5^m, 120 mm, **B** 20^m, 120 mm.

excluded. Measurements of the extinction coefficient at 2780 Å can be performed within about one minute after the end of irradiation but the value of this extinction coefficient does not change during the first hours after irradiation. After 24 hours a slight increase is observed in cases where the irradiation dose had been large and is undoubtedly the result of aggregation. This amount of aggregated material is, however, too small to make any difference in the ultracentrifuge diagram. It is thus evident that the ultracentrifuge diagrams from runs performed within 24 hours after irradiation represent the condition in the solution immediately after irradiation.

As described above, suitable concentrations for studies of the peaks in the ultracentrifuge diagrams with the 12 mm cell used are, for whole molecules 0.05–0.20 %, for half molecules 0.05–0.30 % and for eighths 0.10–0.50 %. A concentration of the irradiated solution of about 0.10–0.20 % is thus suitable for runs in the ultracentrifuge. The peaks of the whole molecules are extremely sharp and rather low scale distances have to be used. On the other hand for the lower concentrations of the halves a rather high scale distance is necessary. It is thus often not possible to get

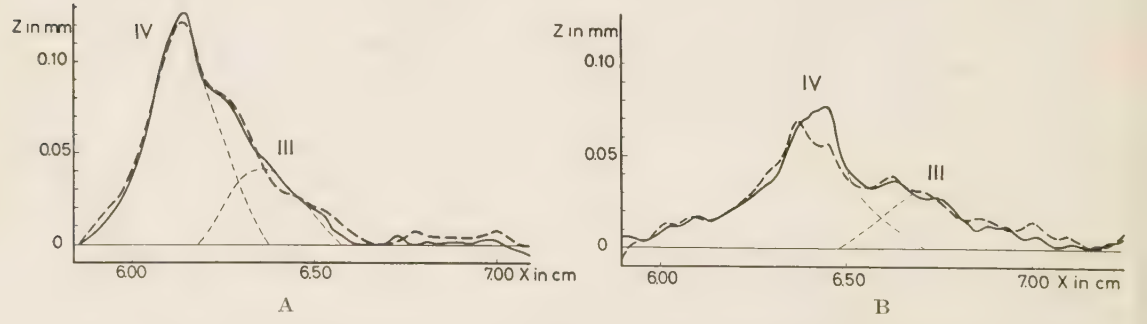


Fig. 39A and B. Ultracentrifuge diagram of hemocyanin 0.19 % at pH 8.2 containing 0.1*M* NaCl irradiated in air with $Q/d_{2537} = 6.9 \times 10^{18}$ $h\nu$/ml. Solid line obtained directly after irradiation, dashed line 72 hours later. Time and scale distance **A** 45^m, 120 mm, **B** 80^m, 120 mm. IV marks the component with $s_{20} \sim 12$.

the concentration of the different components from the same ultracentrifuge diagram at one scale distance only, several scale distances have to be used.

The concentrations from the ultracentrifuge diagrams are determined after 40 min. at full speed, the base line is drawn in the same way as described for unirradiated hemocyanin. For the concentrations used the displacement effect is small enough not to matter.

Irradiation effect on whole molecules

In the present investigation the first experiments were performed at pH 6.2, analogous to Brohult (12). In order to study the first phase of the reaction, solutions 1 %–0.5 % were irradiated and run in the ultracentrifuge at this concentration. Only the half molecules formed were studied at this concentration. It was found that the splitting followed a first order reaction in agreement with Brohult. However, at the highest concentration aggregation started fairly soon and became the main cause of the disappearance of the whole molecules. It was thus immediately clear that lower concentrations at irradiation were desirable if aggregation reactions

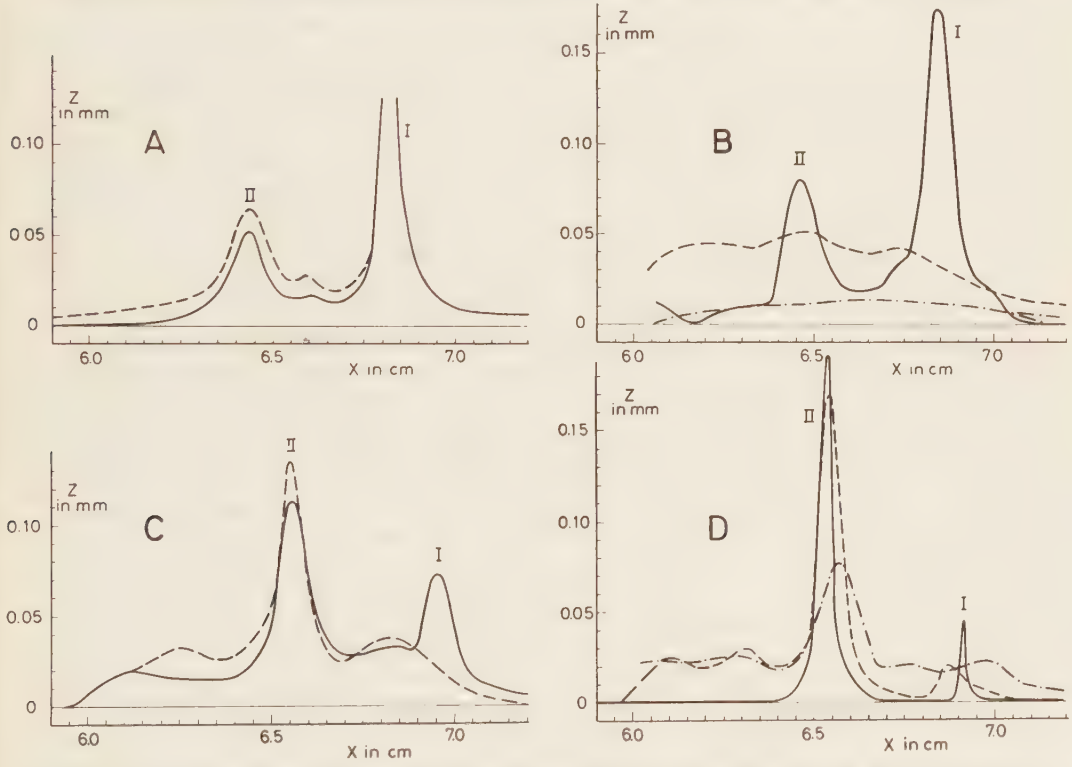


Fig. 40. Ultracentrifuge diagrams for hemocyanin 0.2 % **A** irradiated at pH 6.2 0.1*M* NaCl with $Q/d_{2537} = 5.6$ and 8.4×10^{18} $h\nu$/ml at $dQ/dt = 0.15 \times 10^{18}$ $h\nu$/ml min., **B** with $Q/d_{2537} = 6.9$, 34 and 68×10^{18} $h\nu$/ml, at $dQ/dt = 5.5 \times 10^{18}$ $h\nu$/ml min., **C** with $Q/d_{2537} = 10.9 \times 10^{18}$ $h\nu$/ml. – – – NaCl added to irradiated solution to 1*M*, **D** hemocyanin 0.2 % at pH 5.9 with 1.0*M* NaCl —— unirradiated, – – – irradiated with $Q/d_{2537} = 6.9 \times 10^{18}$ $h\nu$/ml, – · – · irradiated solution dialyzed to 0.1*M* NaCl. Time and scale distance **A**, **B**, **C** 40^{m}, 120 mm, **D** 40^{m}, 80 mm. **A** and **B** are preparations with different quantum yields.

Table 6. Quantum yields for hemocyanin wholes, treated in different ways. $C \sim 0.2$ %, pH 6.2 phosphate, 0.1M NaCl, preparation VII.

Treatment	$\phi \times 10^5$
Undialyzed blood	0.72
Dialyzed blood	0.74
Precipitation with $(NH_4)_2SO_4$	0.78
Dialyzed blood, fraction with 10 % β-component	0.75
Dialyzed blood, fraction with 50 % β-component	0.68

were to be avoided. Preliminary experiments with irradiation at different salt concentrations at pH 6.2 showed that the formation of half molecules decreased with decreasing salt concentration. The forces holding the subunits together are considerably stronger at low salt concentration and we have actually quite different systems to work with. When the concentration of sodium chloride is close to that at which the molecule is split by the salt itself very small doses are needed to cause splitting by light.

In Figs. 49 and 50 $\log c/c^\circ$ has been plotted against the corresponding $Q\,d$ values. For the calculation of the quantum yield see page 42. The absorption coefficient E_a used was 8.0 at 2537 Å.

It seemed suitable to study the irradiation effects on the undissociated molecules in a system containing 0.1M NaCl at pH 6.2 which represents a system of medium stability. Figs. 37 and 40 B show typical cases irradiated in air.

We evidently have here a case where splitting into stable molecules occurs; reaction of the split products with the wholes does not have to be considered, not for lower doses at least. The sedimentation constant of unsplit molecules has not changed markedly, see Fig. 46. In the beginning intermediate and half molecules are seen, later the halves have also been acted upon. After a very high irradiation dose opalescence is observed, and the ultracentrifuge diagram shows a very polydisperse mixture, see Fig. 40. The system thus changes from a paucidisperse system at lower irradiation doses to a polydisperse one at high irradiation doses. In this case the reaction is a one-hit process as far as the whole molecules are concerned. The quantum yields for the disappearance of the wholes varies somewhat from preparation to preparation, $(0.7 - 1.1) \times 10^{-5}$, but not for the same preparation treated in slightly different ways, see Tables 6 and 10.

There is no marked difference for solutions prepared from undialyzed blood and those from dialyzed blood at the low concentrations ~ 0.2 % used. The additional amount of salt in the former solution is evidently too low to matter here. In experiments with solutions containing different amounts of the β-component, 10 % and 50 % respectively, only a deviation of about 10 % in the quantum yield was observed and it seems thus, at least in the case investigated, that the quantum yield is not much different for the two components. This is rather surprising, as one would have thought that inability to dissociate in strong sodium chloride would also mean greater resistance towards the action of light. However, it will later be shown that the wholes at pH 7.6 which in Lontie's view consist of only the β-component, are readily split by irradiation. Solutions that have been frozen show, however, a larger quantum yield than those preserved at + 4 C in the refrigerator. In one case where

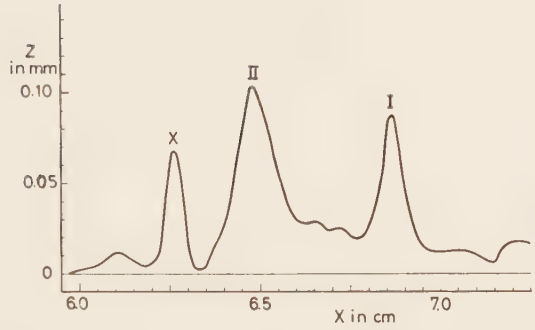


Fig. 41. Ultracentrifuge diagram of a 0.2 % solution of hemocyanin at pH 6.2 with 0.1*M* NaCl irradiated with $Q/d_{2537} = 7 \times 10^{18}$ $h\nu$/ml. Time and scale distance 40^{m}, 120 mm. A peak, X, is seen at 6.26 which has a sedimentation constant of 38.8. This hemocyanin (XI^{c}) had been stored frozen before solutions were made. Time and scale distance 40^{m}, 120 mm.

a solution earlier frozen has been irradiated components corresponding to quarter molecules are found, see Fig. 41.

Table 7 shows the effect of increasing amounts of sodium chloride on the splitting tendency of a frozen preparation which has a relatively high quantum yield.

After high irradiation doses the surface structure has undoubtedly changed markedly and the first stage of denaturation is observed. This naturally makes secondary aggregation reactions more possible and deviation in the quantum yield from the values in the beginning is to be expected.

The question of the appearance of the intermediate products arises. If it is assumed that the molecule is first transformed into intermediates which then split into halves, the concentration of the intermediate products should first increase before any halves are observed; this should then be followed by a slow increase of the halves. Instead a fairly low constant concentration of intermediate products is found. The question whether they represent a small percentage of aggregated halves, or partly split or denatured wholes, cannot be decided. It is rather surprising that the splitting of the wholes is a one-hit process; a multi-hit process involving many bonds or sites necessary for combination seems more reasonable. There must then be some vital bonding mechanism that has to be destroyed before splitting can occur.

Table 7. Quantum yields for hemocyanin, wholes, in solutions with varying amounts of salt. $C \sim 0.2$ %. Preparation XI^{c} stored frozen, and XI unfrozen.

Preparation	Medium	$\phi \times 10^{5}$
XI^{c}	pH 6.2	
	0.04*M* phosphate,	0.20
	0.08*M* ,, ,	0.70
	0.08*M* ,, , 0.1*M* NaCl	1.70
	0.08*M* ,, , 0.2*M* NaCl	3.08
	0.08*M* ,, , 0.4*M* NaCl	4.55
	pH 5.3	
	0.08*M* acetate, 0.1*M* NaCl	0.59
	0.08*M* ,, , 0.2*M* NaCl	0.68
XI	pH 6.2	
	0.08*M* phosphate, 0.1*M* NaCl	1.00

Table 8. Quantum yields for hemocyanin, wholes, influence of buffer. $C \sim 0.2$ %, 0.1*M* NaCl. Preparation VII.

Buffer	$\phi \times 10^5$
pH 5.3, 0.08*M* acetate	0.48
pH 5.3, 0.08*M* phosphate	0.56
pH 6.2, 0.08*M* acetate	0.57
pH 6.2, 0.08*M* phosphate	0.74

Table 9. Quantum yields for hemocyanin, wholes, at different pH's in phosphate buffers containing 0.1*M* NaCl. $C \sim 0.2$ %. Preparation XIV.

pH	$\phi \times 10^5$
5.31	0.58
6.20	0.95
6.71	1.16
7.10	2.42

Table 10. Quantum yields for hemocyanin, wholes, halves, and eighths in different media.

Preparation	Medium	Concentration at irradiation %	$\phi \times 10^5$
	Wholes		
I	pH 6.2, phosphate, 0.2*M* NaCl	1	0.92
I	„ , „ , „	0.5	0.92
XIV	„ , „ , 0.1*M* NaCl	0.1	0.95
XII	„ , „ , „	0.2	1.02
VII	„ , „ , „	0.2	0.74
VII	pH 7.6 acetate, 0.1*M* NaCl	0.2	1.55
VII	0.2*M* $CaCl_2$ (pH $\sim$ 7.2)	0.2	2.11
XIV	pH 7.6 phosphate, 0.2*M* NaCl	0.2	4.02
XI	distilled water	0.2	0.1
	Halves		
XI	pH 7.6, phosphate, 0.2*M* NaCl	0.2	8.0
XIV	pH 5.9, „ , 1.0*M* NaCl	0.2	6.6
	Eighths		
I	pH 8.2, borate-phosphate, 0.2*M* NaCl	0.47	9
XI	„ , „ „ , „	0.2	14

When irradiation is performed at pH 5.3 in acetate buffer with 0.1*M* sodium chloride a lower quantum yield is found (see Table 8). Here aggregation also starts very soon. The forces keeping the units together are of course greater near the isoelectric point and the unsplit excited molecule may either react with similar molecules or aggregate with unchanged molecules. The quantum yield for splitting can only be calculated as long as the aggregation reaction can be neglected.

If a solution irradiated at pH 5.3 is brought to pH 6.2 by means of dialysis it is found that the splitting becomes greater. It is, however, not as great as if irradiation was performed at pH 6.2. If the reverse is done more splitting is seen at pH 5.3 than if the irradiation had been performed here. For some of the still seemingly unchanged molecules at pH 5.3 the binding mechanism operating at pH 6.2 is destroyed and for some of the split molecules at pH 6.2 the binding mechanism acting at pH 5.3 is not destroyed. A completely satisfactory explanation of all these phenomena cannot be given at the present time. It is further found that the quantum yield in phosphate buffer at pH 5.3 is greater than in the corresponding acetate buffer; the same is true at pH 6.2. It is evident that hemocyanin is more stable in the acetate buffer.

Irradiation experiments in a phosphate buffer at pH 5.31, 6.20, 6.71 and 7.10 show a general increase of the quantum yield as is to be expected when the end of the stability region is approached. See Table 9.

In a few cases irradiations at pH 4.8 and 3.6 were performed; the quantum yield for the wholes was here of the same magnitude as for irradiation at pH 6–7. As already mentioned, the diagrams showed a higher degree of inhomogeneity.

Irradiation at pH 7.6, where both wholes and halves are present before irradiation, also shows a one-hit process for the splitting of the wholes. The quantum yield is higher than at the lower pH values, see Table 10 and Fig. 54.

The possible effect of perfluoro-octanoic acid on the stability of the wholes was investigated. According to Klevens (private communication) such acids have a stabilizing effect on proteins in the sense that the latter become less sensitive to heat denaturation for instance. Addition of different amounts of perfluoro-octanoic acid did not prevent the splitting into halves at pH 7.6 and at pH 6.2 there was no decrease in the quantum yield. Perfluoro-octanoic acid thus does not seem to have a stabilizing influence on the forces acting in the above cases. The concentrations of perfluoro-octanoic acid were 0.05–0.005 % in a 0.2 % hemocyanin solution.

Irradiation at low salt concentration at pH 5.3 and 6.2, Fig. 38 (only the buffer solution present at the irradiation) shows that the tendency to aggregation is very much increased especially at 5.3 where very little splitting occurs. When the conditions for splitting are reduced we thus often observe aggregation.

In salt solutions at pH's 7 to 8 containing Ca-ions the wholes remain undissociated; however, at irradiation a very high quantum yield shows that the subunits are not very strongly bound together, see Table 10.

Irradiation of wholes in distilled water at pH 7.1–7.3 showed a low quantum yield. The main peak is, however, considerably broadened and at higher irradiation doses products with lower sedimentation constants are observed (Figs. 42, 53). When the irradiated solution is brought to pH 6.2 some splitting into halves is observed for the smaller irradition doses, for higher irradiation doses the difference between the ultracentrifuge diagram obtained for the solution in distilled water and that in buffer at 6.2 becomes smaller. For very large irradiation doses no change is ob-

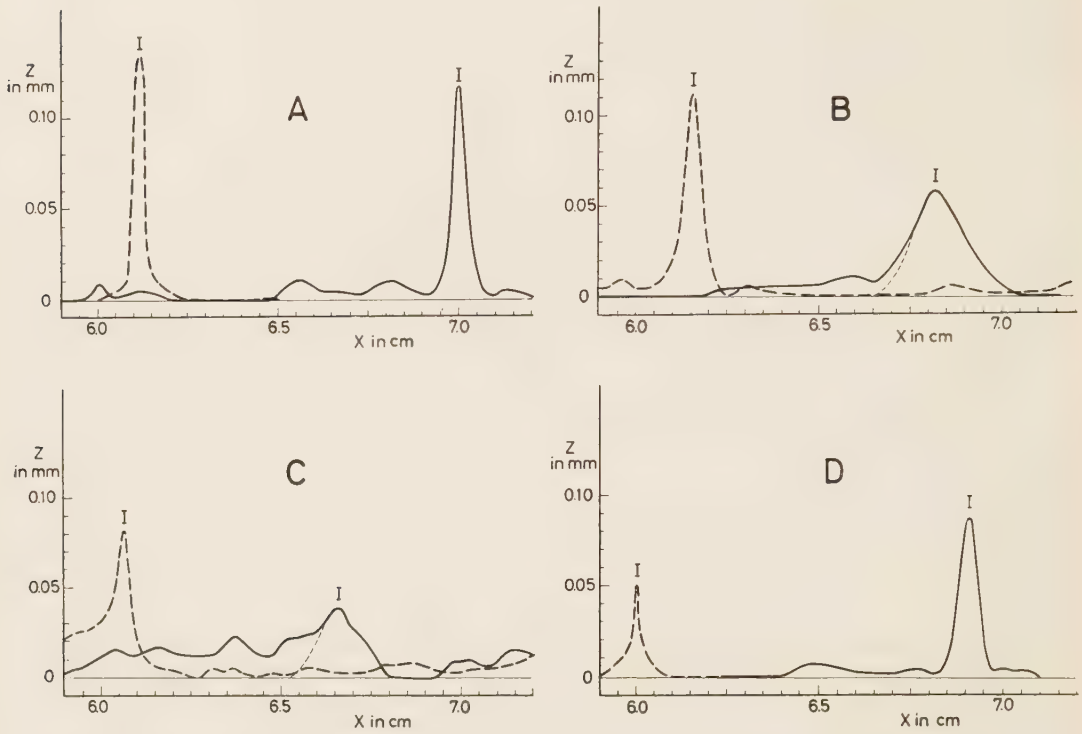


Fig. 42. Ultracentrifuge diagrams of 0.10 % solutions of hemocyanin in H_2O irradiated in H_2O with Q/d_{2537} values of **A** 6, **B** 29 and **C** 58×10^{18} $h\nu$/ml. **D** irradiated in 1 % solution with $Q/d_{2537} = 7 \times 10^{18}$ and then diluted to 0.2 %. Time and scale distance **A** 10^m, 20 mm and 50^m, 60 mm, **B** 10^m, 60 mm and 40^m, 120 mm, **C** 10^m, 60 mm and 40^m, 120 mm, **D** 10^m, 10 mm and 40^m, 20 mm.

served even when the solution irradiated in distilled water is investigated in a medium containing phosphate buffer at pH 5.9 with 1*M* sodium chloride where otherwise splitting of the wholes occurs. Nor is any change observed in the usual buffer at pH 8.2, where normally eighths would have appeared, Fig. 43. It is evident that the molecules have lost their ability to dissociate, we may say that we have encountered a case of internal aggregation or perhaps cross linking. The analogy with the cross linking of synthetic high polymers resulting from irradiation with ionizing irradiation cannot be drawn too far but some kind of network strengthening must have taken place inside the molecules. The forces keeping the subunits together in distilled water are naturally very strong and the energy seems to be used up for other purposes than splitting. If the solution contains even very small amounts of salt at the pH 7.1–7.3 splitting of the wholes occurs.

The internal aggregation is also observed to a small extent at the above mentioned pH's, 5.3 and 6.2. Addition of sodium chloride does not cause the same degree of splitting, in particular the intermediate products seem unaffected, see Fig. 40 C. Bringing the solution to pH 8.2 does not produce only eighths or lower products, some of the higher components remain undissociated. The effect of internal aggregation at pH 6.2 is lower than at pH 5.3 and for small irradiation doses at the

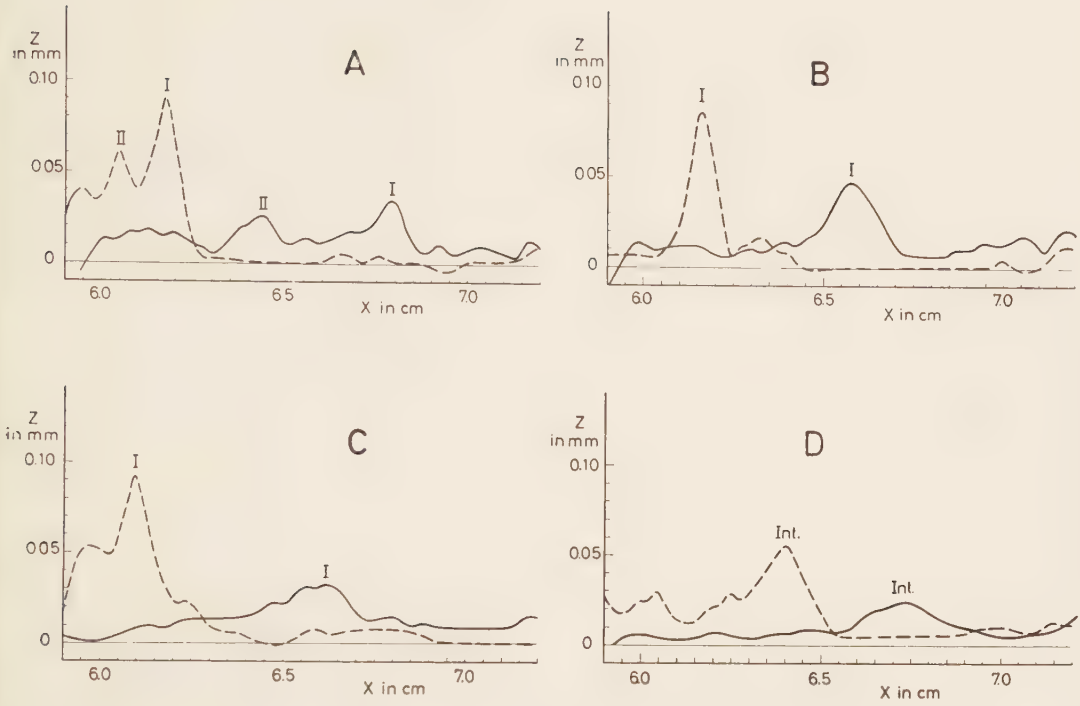


Fig. 43. **A** solution 42**A** brought to pH 6.2 0.1*M* NaCl, **B** solution 42**B** brought to pH 6.2 0.1*M* NaCl, **C** solution 42**C** brought to pH 5.9 1.0*M* NaCl and **D** solution 42**C** brought to pH 8.2 0.1*M* NaCl. Time **A** and **C** 10^m and 40^m, **B** 10^m and 30^m, **D** 10^m and 21^m. Scale distance 120 mm in all cases. **D** 36,000 r.p.m., the others at 24,000 r.p.m. "Int." means intermediate products.

former pH a nearly complete dissociation at pH 8.2 is observed. Besides the eighths a considerable amount of the lower products with sedimentation constant 12 is found. This indicates that the binding mechanism inside the eighths is partly destroyed also when they are parts of the larger units, the wholes and the halves.

The copper band at 3460 Å decreases at irradiation with 2537 Å, see Fig. 56. The quantum yield for a solution irradiated in distilled water was 1.7×10^{-5}.

In order to study energy transfer from the copper band chromophores a solution of whole molecules at pH 6.2 was irradiated at a wavelength ~ 3500 Å. The experimental arrangement was different from that previously described. A high pressure mercury vapour lamp with an appropriate filter was used and the light entered the cuvette containing the solution from above. The stirring was performed by a magnet. No special reference cuvette was used but the irradiation doses were measured before and after the irradiation of the hemocyanin solution by means of the usual uranyl–oxalate actinometer solution. Irradiation doses of about the same magnitude as those used for $\lambda = 2537$ Å were used but no effect could be seen in the ultracentrifuge diagrams. When the solution was brought to pH 8.2 a noticeable amount of the lower product was observed, Fig. 44. It seems thus that the binding mechanism that keeps these low subunits together is the only one affected by $\lambda = 3500$ Å (except of course the copper band itself). A possible explanation is that the bonds

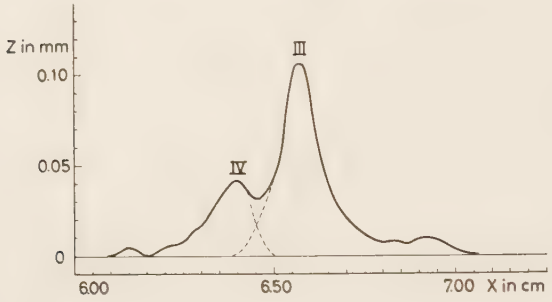


Fig. 44. Ultracentrifuge diagram of 0.37 % hemocyanin irradiated at $\lambda = 3500$ Å with $Q/d_{3460} = 31 \times 10^{18}$ at pH 6.2 containing 0.1*M* NaCl and then dialyzed to pH 8.2 containing 0.1*M* NaCl. Time and scale distance 80^{m}, 40 mm, 36,000 r.p.m. IV marks the component with $s_{20} = 12$.

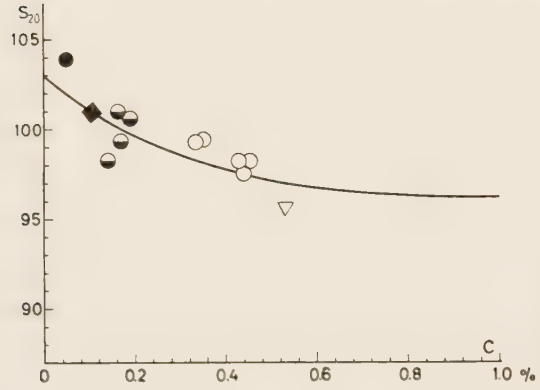


Fig. 45. Concentration dependence of sedimentation constant for irradiated whole molecules. The different points represent various preparations in phosphate buffer at pH 5.3 containing 0.1*M* NaCl. The curve is the same as in Fig. 29A.

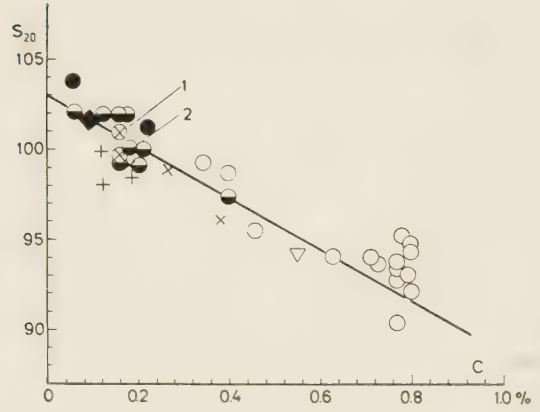


Fig. 46. Concentration dependence of sedimentation constant for irradiated whole molecules. The different points represent various preparations in phosphate buffer pH 6.2 containing 0.1*M* NaCl. Point ⊕ 1 and 2 contains 50 % and 10 % of β-component resp. The line is the same as in Fig. 30A.

involved here are the weakest bonds existing in the molecule, another explanation is that these particular bonds are connected with the absorbing group in some way facilitating energy transfer. The energy at 3500 Å is smaller than at λ 2537 Å and this may explain why no splitting into halves occurs, the binding mechanism involved in the latter case being too strong.

No quantum yield for the destruction of the copper bond is given due to the difficulties with corrections for light scattering effects in this case; it is however certainly lower than for irradiation at λ 2537 Å.

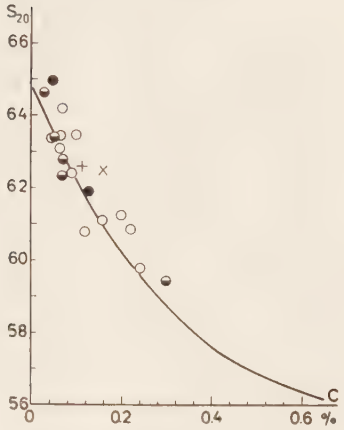


Fig. 47. Concentration dependence of sedimentation constant for irradiated half molecules. The different points represent various preparations in phosphate buffer at pH 7.6 containing 0.2*M* NaCl. The curve is the same as in Fig. 33A.

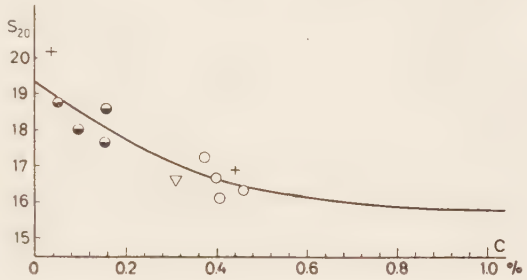


Fig. 48. Concentration dependence of sedimentation constant for irradiated eighth molecules. The different points represent various preparations in borate buffer at pH 8.2 containing 0.2*M* NaCl. The curve is the same as in Fig. 34A.

The different fractions of a solution containing only wholes, obtained by means of frontal analysis on active carbon, and which unirradiated had identical ultracentrifuge diagrams (page 61), were irradiated under identical conditions. The ultracentrifuge diagrams were the same within experimental errors. Frontal analysis was also performed on an irradiated solution at pH 6.2. The first fraction contained practically only wholes which then seem to have lower adsorption than the halves formed. Whether surface effects or size effects are responsible for this is not clear.

Summarizing, the action on the whole molecules is of several kinds: *A*. Splitting of bonds or destruction of binding mechanism, (*a*) occurring inside the molecule with no change in molecular weight, and (*b*) giving rise to splitting into observable subunits; *B*. aggregation, (*a*) internal, resulting in a molecule not showing the normal salt and pH dissociation reactions, and (*b*) external, i.e. aggregation between different units.

As to which of these reaction mechanisms will dominate depends entirely on the strength of the forces keeping the subunits together at the beginning. Aggregation reactions are mostly observed for very stable systems, otherwise splitting. Internal splitting is observed also for irradiation at λ 3500 Å; no external splitting was observed here at pH 6.2.

The irradiation effect on half molecules

The irradiation effect on the halves was studied at pH 7.6 and also in 1*M* sodium chloride with the previously mentioned buffers of pH 5.3 and 6.2, see Figs. 40 and 47. At pH 7.6 eighths are formed and the reaction is a one-hit

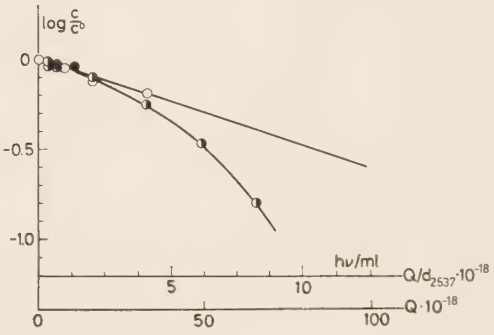


Fig. 49. The decrease of whole molecules upon irradiation at pH 6.2, 0.2*M* NaCl, C = 1 %, ○ nitrogen, ◑ air, ● oxygen atmosphere. $\phi = 0.92 \times 10^{-5}$.

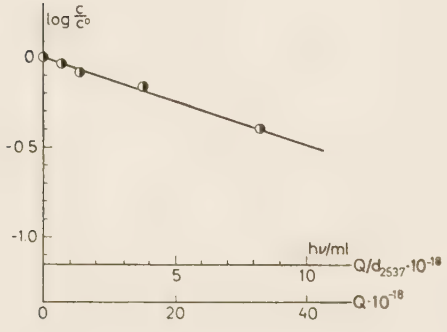


Fig. 50. The decrease of whole molecules upon irradiation at pH 6.2, 0.2*M* NaCl, C = 0.5 %, air. $\phi = 0.92 \times 10^{-5}$.

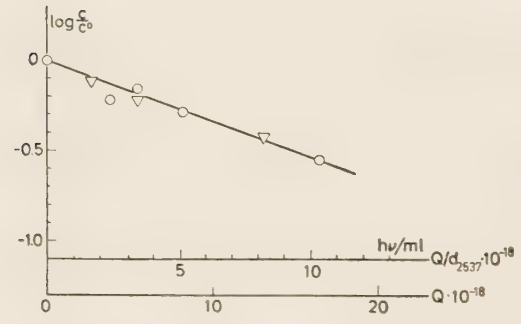


Fig. 51. The decrease of whole molecules upon irradiation at pH 6.2, 0.1*M* NaCl, C = 0.2 %, nitrogen. Intensity dQ/dt ○ 0.55, ▽ 5.5×10^{18} $h\nu$/ml min. $\phi = 1.02 \times 10^{-5}$.

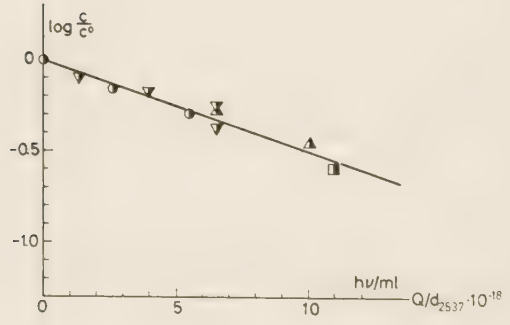


Fig. 52. The decrease of whole molecules upon irradiation at pH 6.2, 0.1*M* NaCl, in air at different intensities $dQ/dt \times 1/d_{2537}$ ◗ 0.1, ▲ 1.8, ▽ 3.6, ◨ 6.9×10^{18} $h\nu$/ml min. $\phi = 0.95 \times 10^{-5}$.

process in analogy with the wholes. The quantum yield is considerably higher than that for the wholes, see Table 10. In strong sodium chloride well defined eighths are not observed, a number of polydisperse products intermediate between halves and eighths is seen. On dialysis against a medium of the usual lower salt concentration (0.1 M) recombination occurs to a very small extent, Fig. 40. The special structure of the halves necessary for this seems to have been destroyed though the molecules still have unchanged molecular weight. The quantum yield in the strong salt medium is $\sim 6.6 \times 10^{-5}$. Studies on the splitting of the halves in the media at pH 5.9 and 5.3 in 1.0 M sodium chloride is difficult as aggregation reactions complicate the picture and the quantum yield cannot be determined with high accuracy.

Studies at the acid pH's are as mentioned above complicated by the inhomogeneity observed.

The irradiation effect on eighth molecules

The eighths upon irradiation give a component with $s_{20} \sim 12$, which may be composed of a sixteenth if the relation $s = k \times M^{\frac{2}{3}}$ can be applied (127), Fig. 39. As in the other cases the reaction is a one-hit process and the quantum yield is higher than for the splitting of the halves, see Table 10 and Fig. 55.

The sedimentation constant for the unsplit eighths does not change upon irradiation, see Fig. 48.

Frontal analysis of irradiated eighths shows that the eighths themselves are more adsorbed than the subunits formed. This experiment was used for the investigation of the changes in light absorption of the different molecular species in the mixture (see Table 3).

From previously described experiments with the wholes it also seemed as if the forces keeping units of the component with $s_{20} = 12$ together were rather weaker than at pH 6.2 where the subunits were part of the large undissociated molecule. In looking for an answer to all these complicated questions about the reaction mechanism one also has to consider the relative importance of the energy transfer in molecules of different size.

Discussion

There has been no evidence in any of the experiments for a dependence on light intensity (the range used for dQ/dt has been $0.15 - 5.5 \times 10^{18}$ $h\nu$/ml). It is of course possible that experiments with very much increased light intensity would reveal that the molecules are sensitive to a time depending multi-hit process. Figs. 51 and 52.

Most of the experiments have been carried out in air but some have been performed in oxygen and nitrogen. There is very little difference between the results in air and oxygen and the same is true in nitrogen atmosphere in those cases where splitting is the dominating reaction. For systems where aggregation occurs, the formation of very large aggregates is favoured in nitrogen atmosphere. Aggregation to large aggregates in nitrogen atmosphere is often encountered for synthetic polymers where it is a sign of chain polymerization which often is prevented by oxygen. The author will, however, not venture to suggest that this is the explanation also here. The interaction with oxygen between an activated complex of molecules preventing aggregation has also been treated by Förster (89).

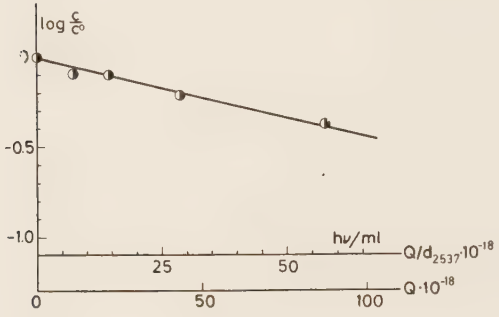


Fig. 53. The decrease of whole molecules upon irradiation in H_2O, C = 0.19 %, air. $\phi = 0.1 \times 10^{-5}$

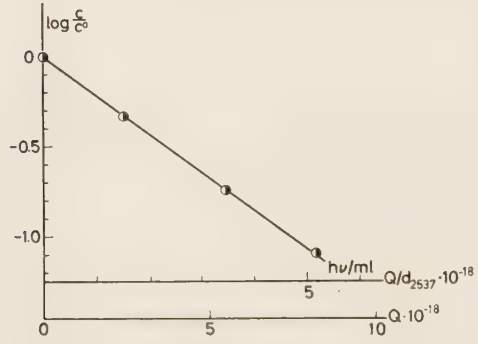


Fig. 54. The decrease of whole molecules upon irradiation at pH 7.6, 0.2*M* NaCl, C = 0.2 %, air. $\phi = 4.02 \times 10^{-5}$.

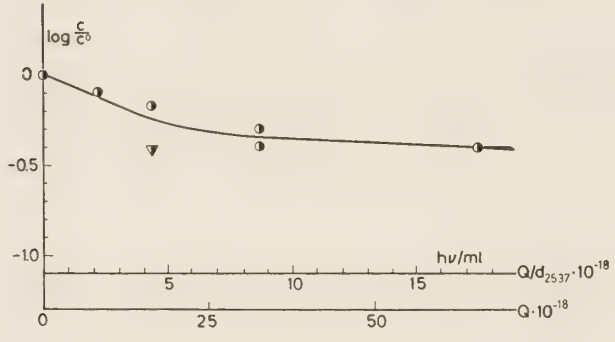


Fig. 55. The decrease of eighth molecules upon irradiation at pH 8.2, 0.2*M* NaCl, C = 0.47%, air. ▽ measured one week after irradiation, other points within 24 hours. $\phi = 8.7 \times 10^{-5}$.

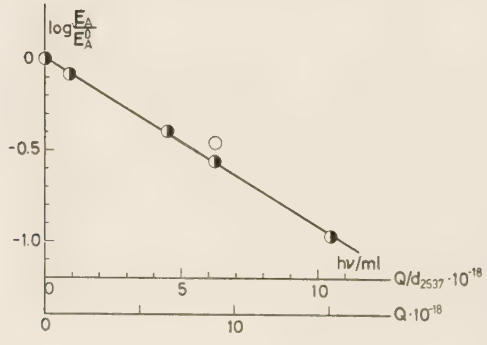


Fig. 56. The decrease of the copper band absorption (E_a) at 3460 Å upon irradiation with λ 2537 Å in H_2O, $C = 0.19$ %, ◑ air, ○ nitrogen. $\phi = 1.7 \times 10^{-5}$.

The irradiations were all performed at 21°C except in a few cases when the temperature was +4°C, the effect was the same in these cases in analogy with the experiments by Svedberg and Brohult (11). As all the centrifugations are run at about 20°C changes due to more or less pronounced secondary aggregation at the irradiation temperature will be difficult to discover.

Electron micrographs of irradiated material are seen in Fig. 26.

It has been found that for both wholes, halves and eighths splitting into subunits is the main reaction upon irradiation. The specific surface structure that ordinarily makes the subunits keep together has evidently been destroyed, possibly by energy transfer. It is not known if –CO–NH– bonds or –S–S– bonds are involved. When the stability of the molecules is decreased, by a change in pH or by addition of salt, an increased quantum yield for splitting into subunits upon irradiation is also found. The effects are, as described, in all cases one-hit processes, and the quantum yield seems in each case to be fairly constant except for the eighths. The constant quantum yield is in agreement with the reasoning in chapter III where it was suggested that the rather small changes in light absorption for hemocyanin, for the irradiation doses employed here, would not markedly affect the distribution of the energy absorbed in a molecule. A multi-hit process might well have been expected involving several different points of attachment between the subunits. It seems, however, as if the destruction of some vital attachment mechanism would be sufficient to separate the subunits. As the splitting decreases when a solution is dialyzed to a pH where the stability is greater, the attachment mechanism may not be completely destroyed. However, an increased tendency to aggregation after irradiation may also explain this. Aggregation is also found and can occur both internally and externally depending on the experimental conditions.

The quantum yields found are too high to agree with the inverse molecular weight relation proposed by McLaren *et al.* (66) for inactivation of enzymes. This applies also to the destruction of the copper band at 3460 Å. However, Kleczkowski (8) found, as already mentioned, that the decrease of enzyme activity of chymotrypsin was slower than the decrease in stability, measured as stability when exposed to 37°C.

A quantum yield of 1×10^{-5} corresponds to an absorption of, on the average, 10 quanta per aromatic chromophore when a splitting occurs.

Part 2. Bovine serum albumin

Earlier investigations on serum albumin

Pedersen (9) noticed that serum albumin at pH 3.5 when irradiated with ultraviolet light showed components sedimenting with approximately twice the rate of the original material.

Sanigar *et al.* (10) who also irradiated serum albumin (human) with ultraviolet light both at pH 7.4 and at pH 5.4 (the isoelectric point is 4.9) in air at 30°C found when the solutions were investigated in the ultracentrifuge that both material sedimenting with a sedimentation constant very close to that of normal serum albumin and aggregates appeared. The effect of the irradiation is thus aggregate formation. No components that sedimented at a lower rate than serum albumin were observed. The aggregation was more pronounced at pH 5.4 and the size of the aggregates

was also larger here. Finally precipitation occurred. Not more than about 5 % of low molecular substances was found. No difference in the ultracentrifuge diagram was observed for irradiations performed at temperatures around 0°C and at 30°C. The ultracentrifuge runs were performed at 21°–26°C. Thus the aggregation reaction must either take place at the same rate at the lower temperature or very rapidly when the solution is brought to the higher temperature.

Svedberg and Brohult (11, 12) after irradiating serum albumin at −180°C found no aggregation at all when the solution was brought to 20°C and investigated by means of ultracentrifugation. However, the irradiated serum albumin coagulated at a lower temperature than usual showing that some change, probably the first stage of denaturation, had occurred.

In the investigations described, the light from the whole ultraviolet spectrum had been used. Rideal and Roberts (13), however, used practically monochromatic light of wavelength 2537 Å when they irradiated bovine serum albumin. The changes in the molecular weight were then studied by osmotic pressure measurements. Irradiations were performed at pH 6.0 and 7.2 and at rather high ionic strength usually 0.4. The concentration was 1 %.

They found that the irradiated solutions showed a marked increase in the mean molecular weight. Up to a certain irradiation dose the reaction was the same in oxygen and in nitrogen, then the mean molecular weight started to decrease in oxygen whereas it continued to increase in nitrogen. Rideal and Roberts assumed that aggregation was the only result of irradiation performed in nitrogen. In oxygen aggregation first takes place whereas splitting occurs at a later stage.

A greater increase in molecular weight was found at pH 6.0 than at pH 7.2. The above authors plotted the mean molecular weight against the irradiation time or dose and took the slope of this curve as a measure of the aggregation rate. As the slope of this curve was very low at small irradiation doses they assumed that induction periods were always present. From the expression for the number average molecular weight, where $\bar{\phi}$ is an average quantum yield, compare page 46,

$$M_n = \frac{N^0 M_1}{N^0 - \bar{\phi} Q}$$

we see that if M_n is plotted against Q a hyperbola is obtained. The slow increase at first is quite natural. Initially ϕ is the same as the quantum yield for the action

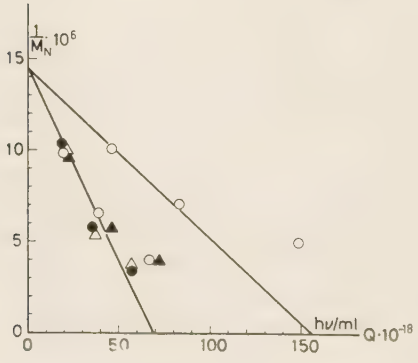


Fig. 57. $1/M_N$ as a function of Q from Rideal's values (13). Left curve pH 6.0, right curve pH 7.2. The points are marked as in (13). $C = 1$ %. Value of dQ/dt is 0.09×10^{18} $h\nu$/min. for ○ ▲ and 0.04×10^{18} $h\nu$/min. for ● △.

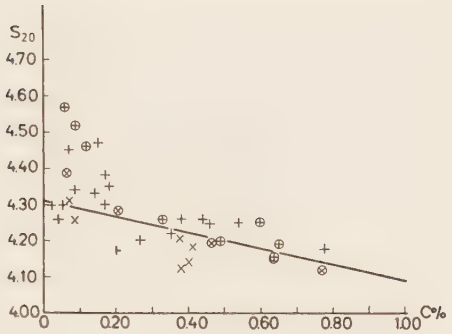


Fig. 58. Concentration dependence of sedimentation constant for bovine serum albumin molecules ⊕ ⊗ unirradiated at pH 6.0 and 6.2 resp. + × irradiated at pH 6.0 and 6.2 resp.

on the original serum albumin molecules. If $\bar{\phi}$ decreases with the increasing molecular weight, which is highly probable, see page 46, a marked deviation from the hyperbolic form will be observed. This is probably the reason why a straight line could be obtained for the relationship between M_n and Q by Rideal and Roberts. If instead, $1/M_n$ is plotted against Q a straight line with a slope proportional to the quantum yield is obtained. Fig. 57 shows such curves, where the data of the above authors have been used. For mean molecular weights $> 200{,}000$ a deviation is seen, the quantum yield then probably starts to decrease. The curves represent experiments at two different intensities. If the reaction would be dependent on the intensity, different ϕ-values would be obtained. This is not the case and the aggregation rate is thus direct proportional to the light intensity.

For the reaction mechanism Rideal and Roberts and earlier Roberts (113) propose a splitting of the serum albumin molecule at the peptide bond into two large radicals which immediately react with –OH and –COOH groups on the intact protein. The evidence for reaction with these groups was derived from the fact that the mean molecular weight for the irradiated serum albumin decreased when acetic acid, providing –COOH groups, was present instead of KCl in the buffer system and that the same happened when –OH groups in the form of hydroquinone and tyrosine were added. The macroradicals were then assumed to react also with the low molecular compounds. The presence of thiol and amino groups had no influence and thus these groups were considered not important in the aggregation reaction. As no determinations of absorption spectra either of unirradiated or irradiated solutions are reported one is doubtful about the evidence in the solutions containing hydroquinone and tyrosine. The latter substances show rather strong absorption at $\lambda = 2537$ Å and corrections must be applied for their light absorption otherwise the effect of irradiation will seem to be too low.

The occurrence of split products in a solution irradiated in oxygen is explained by reaction between the macroradicals and hydroxyl radicals which are assumed to be formed from water and atomic oxygen. The result will be –COOH and –NHOH groups.

For a case of splitting into two large radicals the aggregation rate is proportional to the square root of the intensity if the reverse reaction is dominant whereas it is directly proportional to the intensity if the forward reaction is dominant (13). The slopes of the curves plotted by Rideal and Roberts gave values that showed that the aggregation rate was proportional to $I^{0.55}$, where I is the intensity. From the curve where $1/M_n$ is plotted against Q it seems more probable that direct propor-

tionality against the intensity holds. This makes the proposed reaction mechanism less probable as the reverse reaction must be the dominating reaction if the highly improbable splitting into two macroradicals occurs at all. The Franck-Rabinowitch effect must be extremely important in this case.

The presence of radicals in the solution was demonstrated by Rideal and Roberts. Acrylonitrile present in the serum albumin solution irradiated in nitrogen was completely polymerized. Chemiluminescence with luminol was also observed.

Present investigation

It seemed of interest to study serum albumin solutions irradiated under rather similar conditions as those used by Rideal and Roberts and if possible combine their osmotic pressure data with data from sedimentation experiments.

A detailed description of serum albumin is given in (112).

Material used

Crystalline bovine serum albumin prepared by Armour was used in all experiments. Two different preparations were used but they differed very slightly from each other. Approximately 90 % of the protein sedimented in the ultracentrifuge with the sedimentation constant characteristic for serum albumin, the rest consisted of aggregation products, most of which sediments with a sedimentation constant corresponding to twice the molecular weight of the ordinary serum albumin, see Fig. 59. The tendency to form aggregation products is very great and it is not unusual to find that 20–30 % of a preparation is aggregated. The preparations used must then be considered as reasonably good.

Experimental

The concentration was determined by measuring the refractive index on a concentrated solution, which was subsequently diluted. For the refractive index increment the value 189×10^{-5} at 5460 Å (128) was used. The optical density at 2780 Å is also a good measure of the concentration. The value of the extinction coefficient is determined for a solution where the concentration has been measured refractometrically. For $E^{1\,\%}_{1\,\mathrm{cm}}$ cm the value 6.54 was found as a mean value of a number of determinations (compare chapter I). The two different preparations did not differ in extinction coefficient. It is of course necessary in this connection to use solutions with very low light scattering, see chapter I.

The spectra of both unirradiated and irradiated serum albumin are treated in chapters I and III.

Both apparatus 1 and 2 were used and the experimental arrangements at irradiation were as in chapter II.

The buffers used were the same as those used by Rideal and Roberts, that is at pH 7.2 0.0533*M* Na_2HPO_4, 0.0133*M* KH_2PO_4 and 0.3*M* KCl, ionic strength 0.4 and at pH 6.0 the same buffer adjusted to this pH with HCl. Buffers with pH values 5.8, 6.2 and 8.2 of the same composition as described earlier for hemocyanin were also employed.

Sedimentation behaviour of unirradiated serum albumin

For the ultracentrifugation a 12 mm cell and a speed of 60,000 r.p.m. were used. As serum albumin has a high diffusion constant the peaks are rather broad and there is no difficulty in getting good values for the concentration from the ultracentrifuge diagram. Lower concentrations than 0.1–0.2 % seemed not suitable as the boundaries have a tendency to become rather unstable then. The concentration dependence for the s_{20} values is seen in Fig. 58. To the observed s_{20} values the concentrations corresponding to the x^m values are taken, see page 50. The line is drawn in the way now agreed on by most of the workers in this field (112), the extrapolated value at zero concentration is then given as 4.30. It is seen that the points at concentrations lower than 0.1 % show large variations and the possibility of a more curved line is indicated. High values for the sedimentation constant at low concentrations were also observed by Kegeles and Gutter (129). The k-value has been calculated from the straight line and is 0.05. Earlier an $(s_{20})_0$ value of 4.70 was used and the corresponding molecular weight was 68,000–70,000, the new value 4.30 gives a considerably lower molecular weight in comparison, 65,000 as reported by Creeth (130). Since the value of the molecular weight for serum albumin has been revised several times of late, and there is no certainty of its correctness, the old value 69,000 has been used for the calculations of the quantum yield.

Sedimentation behaviour of irradiated bovine serum albumin

The irradiations were first performed at pH 6.0 at a concentration of about 1 % in nitrogen atmosphere, in air and bubbled through by oxygen. Fig. 60 shows typical ultracentrifuge diagrams.

Only seemingly unchanged serum albumin and aggregation products are observed. The sedimentation constant of the unaggregated material has not changed, see Fig. 58. Aggregation is thus the obvious effect of irradiation. Centrifugations were performed with the irradiated solution immediately after irradiation, after 3 hours, after 6 hours and after 24 hours. It was found that though the absorption spectrum changed during this time, see Fig. 19, the ultracentrifuge diagram was unchanged. The reactions affecting the molecular weights thus take place at or immediately after irradiation. Very slow secondary aggregation reactions also occur but noticeable reduction in the material and corresponding changes in the ultracentrifuge diagram are not observed until a week or more after irradiation. Upon standing for several months precipitates are always observed.

After large irradiation doses a small amount of material sedimenting at a lower rate than the main peak is observed. This "tail" is larger when irradiation has been performed in oxygen but it never exceeded 10 % of the total material. Even after very large doses no great amount of split products has been observed. Aggregation is the dominating reaction also in oxygen. It is to be noticed that Rideal and Roberts observed the same mean value of the molecular weight for irradiation in nitrogen and in oxygen until a Q value of 20×10^{18} $h\nu$/ml was reached after which a sudden decrease in molecular weight was observed in the case of the solution irradiated in oxygen. As no extensive splitting at this or higher Q values is observed in the ultracentrifuge diagrams it is difficult to explain the reason for the mentioned decrease; the small tail can hardly be responsible for this.

Very small amounts of dialyziable products are formed, only about 1–2 %, as determined both from measurements of the spectra and of the refractive index on

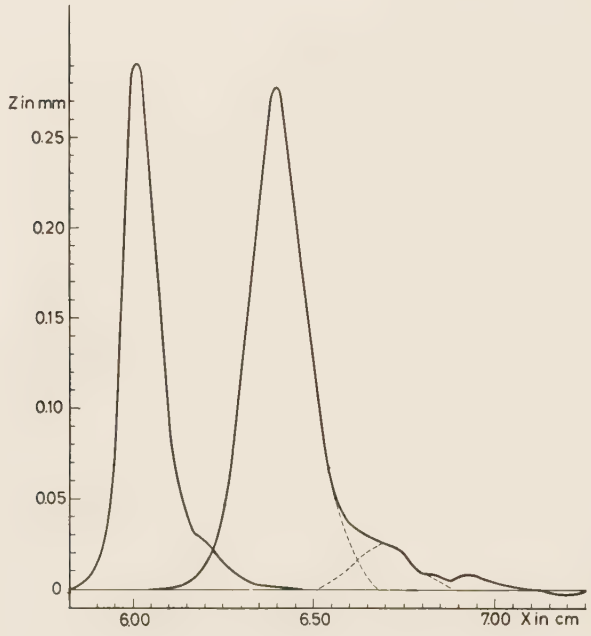


Fig. 59. Ultracentrifuge diagrams for unirradiated bovine serum albumin, 0.90 % at pH 6.0 with 0.1*M* NaCl. Time and scale distance 20^{m}, 20 mm and 80^{m}, 40 mm.

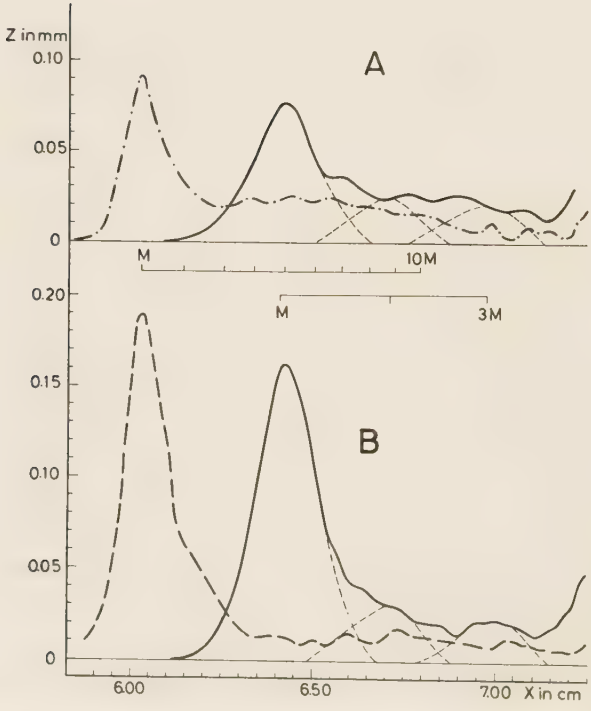


Fig. 60. Ultracentrifuge diagrams of irradiated bovine serum albumin, 0.90 % at pH 6.0. **A** $Q/d_{2537} = 10.2 \times 10^{18}\ h\nu$/ml in nitrogen atmosphere, **B** $Q/d_{2537} = 8.0 \times 10^{18}\ h\nu$/ml in air. Time and scale distance 20^{m}, 20 mm and 80^{m}, 40 mm for both. The positions of molecules with multiple molecular weights are marked in the diagram.

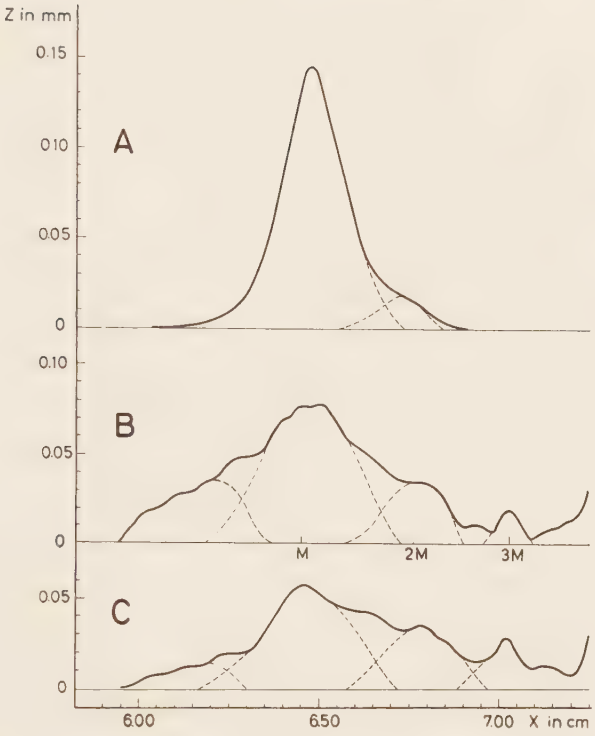


Fig. 61. Ultracentrifuge diagrams for bovine serum albumin 0.2 % at pH 8.2 **A** unirradiated, **B** and **C** both irradiated with about $Q/d_{2537} = 30 \times 10^{18}$ $h\nu$/ml, **B** in bubbling oxygen, **C** in nitrogen. Time and scale distance 80^{m}, 120 mm for all curves. The positions of molecules with multiple molecular weights are marked in the diagram.

both the dialyzed solution and the dialyzate. This is lower than reported by Sanigar *et al.* (10) who gave about 5 %. As already mentioned Roberts (113) reports that for solutions irradiated in oxygen tyrosyl and tryptophyl groups are found in the dialyzate. In the present investigation no special examination of the dialyzate was performed.

In Fig. 60 the positions of the peaks for aggregates with molecular weight $n\,M$ are shown. It has then been assumed that the f/f_0 value is the same, 1.29, as for serum albumin. Lundgren and Williams (127) have given the formulas for this case; the s values are proportional to $M^{\frac{2}{3}}$. In unirradiated serum albumin the small peak sedimenting in front is thus probably a dimer. It is seen that it is possible to assume that multiples of the original molecular weight are present in the irradiated solutions. There does not seem to be preferably more of any particular component; one should perhaps have expected a larger portion of dimers as the probable first aggregation product. At the highest Q values the solution showed a faint opalescence but no precipitation was observed.

We have here a system of many components and the Johnston-Ogston effect might be expected to make the concentrations of the component with lower molecular weight too high. Here we are only interested in the serum albumin itself and it is seen from the formulas that as long as the concentration of this component is large, up to about 50 % of the total material, displacements cannot exceed 10 %; the ratio between the sedimentation constant of intact serum albumin and the next higher component being ~ 0.60. The same result is obtained when a particular

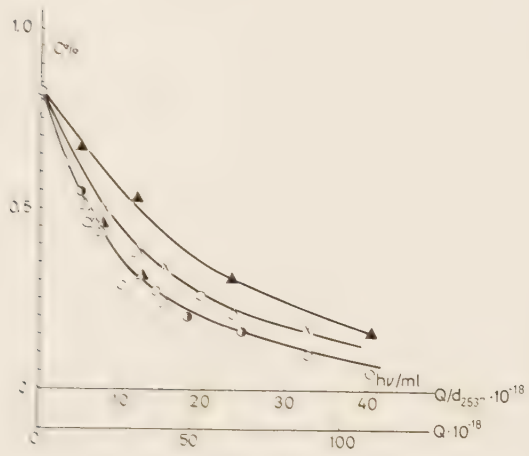


Fig. 62. The decrease of bovine serum albumin molecules upon irradiation, open marks in nitrogen, half filled in air, filled with oxygen bubbling through at pH ○ 6.0, △ 6.2, × in nitrogen at pH 7.2, corresponding intensities $dQ/dt = 1.9$, 0.5 and 0.5×10^{18} $h\nu$/ml min.

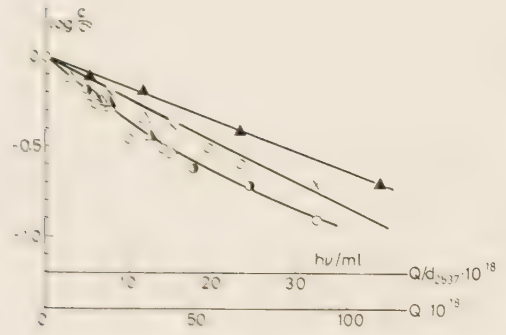


Fig. 63. Legend same as in Fig. 62.

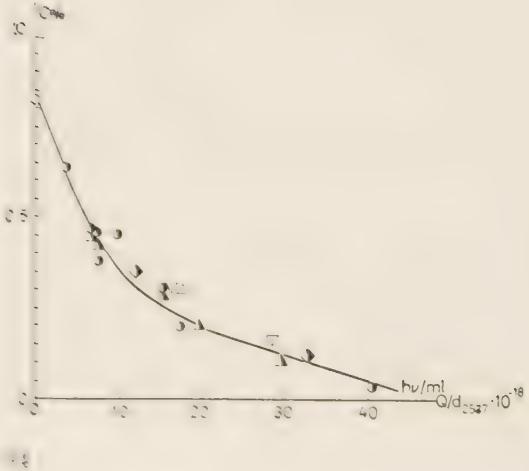


Fig. 64. The decrease of bovine serum albumin molecules pH 6.2 upon irradiation at different intensities dQ/dt. ◇ ▽ 0.15×10^{18} $h\nu$/ml min., ○ △ 5.2×10^{18} $h\nu$/ml min. $c^0 = 0.83$ for ◇ ○; $c^0 = 0.166\%$ for ▽ △, in latter case values plotted are $0.83\ c/c^0$. The curve drawn is the same as in Fig. 62. Atmosphere marked as in Fig. 62.

Table 11. Quantum yields for bovine serum albumin.

Preparation	pH	Atmosphere	$\phi \times 10^3$
I	6.0–6.2	air, nitrogen	2.2
II	6.0–6.2	air, nitrogen	2.2
I	6.0–6.2	oxygen	1.1
I	7.2	air	1.5

irradiated solution is run in the centrifuge at different dilutions. It is thus permissible to calculate the concentration as if no displacement was present, instead of using formula (12), chapter V. In more unfavourable cases centrifugations were performed at a series of decreasing concentrations. For the components with higher n-values it is not possible to calculate the concentrations with any certainty and no conclusion can be drawn about the ratios in which they occur in the mixture.

The concentration, c, of the unaggregated serum albumin was determined after 80–100 minutes sedimentation at full speed. A constant value of the concentration was then reached and the peak represented only unaggregated material. In Figs. 62 and 63 $c \cdot c^0$ and log $(c \cdot c^0)$ are plotted against $Q \cdot d$. c^0 is the original concentration of the unaggregated serum albumin and is 7 10 % lower than the total concentration C (compare the formula page 42). In chapter III it was found that the strong increase in light absorption at $\lambda = 2537$ Å was the same for all the molecules and thus that the molecules with the original molecular weight absorb the same number of quanta per time interval during the whole irradiation if the experiment is performed under conditions where all incident light is absorbed—a condition fulfilled in this investigation. It is seen that the curve for the logarithmic relation is nearly a straight line. We have here a reaction of the one-hit type. Evidently the quantum yield is not much influenced by the increased extinction coefficient; however, the spreading of the points in Fig. 63 is larger than for hemocyanin, Figs. 49–54.

Irradiations performed at different light intensities show furthermore that the reaction is independent of light intensity; that is, the aggregation rate is directly proportional to the light intensity. The same was found from the osmotic pressure measurements by Rideal and Roberts, see Fig. 57. The quantum yield in the latter case is immediately obtained from the slope of the curve according to page 88.

In chapter IV it was shown that if the aggregation reaction took place with an intact molecule, the quantum yield in Fig. 63 would be twice the real photochemical one; if reaction took place between two active molecules the quantum yield in Fig. 63 gives the real value. The quantum yield from Fig. 63 is 0.0022 and from the osmotic pressure measurements 0.0012. It would thus seem that an activated molecule aggregates with an intact molecule. For the curve from irradiation in oxygen the quantum yield is 0.0012. As the above mentioned irregularities were observed for the corresponding osmotic pressures, comparisons cannot be made here. It is difficult to reconcile the data obtained with a splitting into two large radicals, which then cause the aggregation. For the quantum yields, see Table 11.

Experiments were also performed when hydroquinone and tyrosine had been added to the serum albumin solutions before irradiation. No signs of products with sedimentation constants lower than that of the intact material were observed. According to Rideal and Roberts (13) the low molecular substance should have reacted with the radicals which then obviously would show up as components with

lower sedimentation constants. The decrease in aggregation products was as expected due to the decrease in number of quanta absorbed by serum albumin in a mixture also containing other absorbing substances.

Irradiations at pH 7.2 showed small changes compared to those at pH 6.0, at most a decrease of about 30 % could be observed, whereas Rideal and Roberts (13) report that the aggregation rate is 2.5 times as high at pH 6.0 as at pH 7.2. The ratio of the quantum yield derived in the two different ways will in the latter case be close to 3, Figs. 57 and 63. These values are, however, regarded as less certain.

The effect of irradiations performed at +3°C was the same as that at +21°C, the temperature normally used. The temperature at centrifugation was ~ 27–28°C. The aggregation reactions are thus not temperature dependent—in agreement with the observations of Sanigar *et al.* (10).

Intensities were varied from 0.15–5.2 × 10^{18} $h\nu$/ml min. and concentrations from 0.2–1 %. It is evident that there does not seem to exist any definite dependence either on intensity or concentration. If the "life time" of the activated molecule was very short an increase in the number of aggregates should be expected at higher concentrations (89). For the concentration range used such effects seem not to be apparent. That the reaction is a one-hit process seems doubtless.

Irradiations at pH 6.2 at a lower ionic strength showed about the same effect as irradiations at pH 6.0, Fig. 62. However, at pH 5.8 the aggregation was much more pronounced. At this pH perfluoro-octanoic acid was added, just enough to cause a very small aggregation (131). Upon irradiation, the formation of the extremely large aggregates found at pH 5.8 was prevented. This was evident also in the spectrum where a large increase partly due to light scattering was observed for the solution irradiated without perfluoro-octanoic acid. The solution was 0.1 % in perfluoro-octanoic acid and 0.2 % in serum albumin.

Irradiation was also performed at pH 2.2. Here serum albumin has a lower sedimentation constant, 3.2 in the actual case. On dialysis to pH 6.0 the sedimentation constant regained its normal value, the sedimentation diagrams showed, however, that whereas serum albumin at pH 6.0 had only about 7–10 % aggregates the dialyzed products had about 20 %. 2–3 % sedimented as a tail behind the main peak. The medium at pH 2.2 (0.007M HCl and 0.14M KCl) thus affects the surface properties of the serum albumin making it more inclined to aggregate (brings about some kind of denaturation). When the serum albumin solution was irradiated at pH 2.2 there was practically no aggregation for Q/d values around 10×10^{18} $h\nu$/ml whereas the effect at pH 6.0 is quite pronounced. The sedimentation constant also remains unchanged after irradiation. The irradiated solution was dialyzed against the usual buffer pH 6.0 and as a result aggregation products appeared. The concentration of the latter was higher than if irradiation had been performed at pH 6.0 and of course much higher than that reported for the unirradiated solution above. Only about 5 % sedimented as a "tail" of the serum albumin peak. This is an aggregation reaction that can hardly be ascribed to an activated molecule but must depend on some change that has occurred in the molecule, probably in the surface, and which facilitates aggregation close to the isoelectric point where the electrostatic repulsion is lower. These are probably the same kind of changes that are responsible for the lower coagulation point of serum albumin observed after irradiation (see page 88).

Irradiation in the presence of acrylonitrile gave the same result as observed by Rideal and Roberts (13); extensive polymerization was obtained in nitrogen and also to a much lesser extent in air and oxygen. Radicals are thus formed during the

irradiation. As to whether this radical formation is the important reaction for the aggregation cannot be decided, it may just be one of many reactions.

Irradiation at pH 8.2 showed that aggregation occurred at a lower rate here both in nitrogen and oxygen. In the latter case considerable amounts of products sedimenting at a lower rate were observed, see Fig. 61. This is the only case where evidence for appreciable amounts of split products has been found. Reactions with oxygen are generally easier at alkaline pH's and the aggregation is obviously prevented by this. It seems not unlikely that an unfolding resulting from splitting in the weaker bonds will lay the protein molecule open to attacks at the peptide bond. The split products may then be stable molecules with on the average half the molecular weight.

It has been observed by Duggan, Giese and Luck (85) that serum albumin irradiated in the presence of sodium caprylate shows a smaller increase in the ultraviolet spectrum than in the absence of this substance. It was questioned whether the sodium caprylate inhibited the reactions responsible for the change in the chromophores. As viscosity measurements indicated that aggregation was also less pronounced in the solution containing sodium caprylate, the difference in the absorption spectra could either be caused by light scattering or by new chromophores formed at aggregation. In order to investigate this, serum albumin solutions at pH 6.2 and concentration 0.98 % were irradiated in 0.15 % sodium caprylate which has negligible absorption compared to that of the serum albumin. Aggregation occurred also in the presence of sodium caprylate but not to the same extent as in a control solution free of sodium caprylate. In none of these cases was light scattering of importance as shown by the low value at λ 4000 Å, the spectra of the two solutions were within experimental error the same. The increased extinction in the above mentioned investigation is thus undoubtedly due to light scattering, the experimental conditions there were also such that larger aggregates could be expected. The action of sodium caprylate is evidently one that makes aggregation more difficult. The concentration of the sodium caprylate used has no influence on the changes in light absorption.

Urea was added to serum albumin solutions at pH 6.0 both before and after irradiation. Irradiation in the presence of urea did not prevent aggregation altogether but the effect was diminished. Addition of urea after irradiation resulted in dissociation of the very largest aggregates formed, but there was no change in the serum albumin peak itself. The solutions were 1 M in urea.

Incidentally it should be mentioned that Rosen (88) has found that serum albumin subjected to ionizing radiation where the action is the result of radicals formed from the water, shows aggregation reactions that in the ultracentrifuge resemble those in the present investigation. He is of the opinion that two similarily affected molecules react with each other.

Summarizing, we may then conclude that serum albumin when irradiated in nitrogen or air becomes "aggregation reactive". The closer to the isoelectric point the larger the aggregation. The aggregation reaction takes place at the same rate near 0° and 21°C. Aggregation is increased for a solution irradiated away from the isoelectric point when it is brought to a pH closer to this point. This additional aggregation is probably caused by changes in the surface of the same kind that precedes denaturation. The two aggregation effects might actually be the same. The only thing that speaks against this is the fact that serum albumin irradiated in the frozen stage does not aggregate when brought to room temperature.

Different substances such as urea, sodium caprylate and perfluoro-octanoic acid partly inhibit the aggregation reaction either by increasing the electrostatic repulsion or by diminishing the denaturation effects.

If oxygen is present in large amounts the aggregation reaction is also diminished but except at alkaline pH's there is no evidence of products sedimenting at a lower rate than serum albumin itself. It is not certain whether the slowly sedimenting products are serum albumin molecules whose shape has been altered or if they are split products. In the latter case a splitting in the peptide bond and formation of stable molecules may occur. Aggregation also occurs at the same time.

Comparisons with osmotic pressure data indicate that one activated molecule reacts with one unactivated molecule.

The splitting into two macroradicals is not probable.

The presence of radicals is, however, shown by the fact that acrylonitrile polymerizes when present in the irradiated serum albumin solutions.

Summary

The purpose of this work has been to study the changes in light absorption and molecular state of *Helix pomatia* hemocyanin and bovine serum albumin upon irradiation with ultraviolet light λ 2537 Å.

1. The light absorption of the protein chromophores in the ultraviolet region has been described and special attention has been given to the absorption at λ 2537 Å.

2. The influence of light scattering on the protein spectra in the ultraviolet has been briefly dealt with and the corrections to be applied for hemocyanin, which shows appreciable light scattering, have been investigated.

3. Experimental arrangements for irradiation at λ 2537 Å are described. The number of quanta absorbed per ml of solution has been determined by using uranyl oxalate-oxalic acid and monochloroacetic acid as actinometer solutions.

4. The changes in the light absorption of the protein chromophores upon irradiation with λ 2537 Å have been investigated both when they are present in small molecules, such as amino acids, and when they are present in large molecules such as proteins. The changes are not due primarily to oxygen but differences in the appearance of the spectra are observed for solutions irradiated in oxygen and nitrogen.

The most marked changes occur in the aromatic groups. The phenyl group seems to disappear completely and the same is probably true of the pyrrole ring in the tryptophan. The benzene ring is quite likely, as would be expected, more stable, but, as in the case of phenylalanine, a very large increase in light absorption is found.

Chromophores incorporated in proteins exhibit a larger increase in light absorption after irradiation than the corresponding amino acid mixture. The reason for this has been discussed.

When the chromophores are present in large aggregates, as is often the case when irradiation has proceeded for some time in nitrogen atmosphere, the decrease of the protein maximum is not as pronounced as when irradiation is carried out in oxygen.

For the protein molecules investigated the observed changes in light absorption are distributed evenly over the whole material. On the average all the molecules show the same spectra.

Changes in light absorption and in "molecular weight" properties although not necessarily dependent on each other often run together.

5. Formulas for the calculation of the quantum yield have been given under different assumptions. The influence of secondary reactions on the quantum yield has been discussed.

6. The change in molecular state upon irradiation has been studied by means of ultracentrifugation. Therefore concentration dependent sedimentation in systems of one and several components and its implications on the determination of sedimentation constants and concentrations have been treated.

7. A few electron micrographs of *Helix pomatia* hemocyanin, both unirradiated and irradiated, have been made.

8. The sedimentation behaviour and stability of unirradiated *Helix pomatia* hemocyanin have been investigated in different buffer systems. Where possible, comparisons are made with earlier work and the agreement is generally good.

9. From the experiments with irradiated hemocyanin it is concluded that the sedimentation constant of unsplit molecules in irradiated solutions is, within experimental error, the same as in unirradiated solutions.

In all cases a one-hit process is involved. When the undissociated molecule is irradiated in a system where the stability, that is the binding of the subunits, is not too great, a splitting into halves is found. The quantum yield for this is about 1×10^{-5}. The quantum yield increases with salt concentration and increases with pH, when going away from the isoelectric point. Hemocyanin irradiated in distilled water shows a very much smaller quantum yield $\sim 0.1 \times 10^{-5}$. In a region where an unirradiated solution is partly dissociated the quantum yield for the splitting of the undissociated molecules increases to 4×10^{-5}.

The halves upon irradiation yield eighths and lower products. The quantum yield is about 8×10^{-5}.

The eighths upon irradiation give a component sedimenting with $s_{20} \sim 12$. This component may be a split product of half the molecular weight. The quantum yield is about 11×10^{-5}.

The splitting is independent of temperature and of the atmosphere in which irradiation is performed.

The splitting is partly reversible, i.e. split products formed in a system of lower stability may recombine in a system of higher stability.

Parallel to the splitting of weak bonds, which seems to be the first effect of irradiation, some kind of internal formation of new bonds (internal aggregation) takes place. This is evident from the fact that irradiated molecules do not show the same degree of either salt or pH-dissociation.

In more stable systems, e.g. at low salt concentration and at pH's near the isoelectric point, aggregation reactions are also found. The same is true in all systems where irradiation has proceeded for a very long time. The aggregation reactions have, however, not been studied in greater detail.

The copper band at 3460 Å disappears upon irradiation at λ 2537 Å, $\phi = 2 \times 10^{-5}$. Irradiation which principally consists of λ 3500 Å has the same effect but the quantum yield is much lower. No splitting into halves occurs for irradiation at 3460 Å. However, when the solution is dissociated into eighths a considerable amount of the component with $s_{20} = 12$ is observed. This suggests that some internal change has occurred.

Energy transfer quite likely plays an important role in all the experiments with hemocyanin described above.

10. Investigations of the sedimentation behaviour of unirradiated bovine serum albumin have been performed. Agreement is found with results reported from other laboratories. The sedimentation constant of unaggregated material is the same as for unirradiated serum albumin.

Serum albumin aggregates upon irradiation. The aggregation mechanism is approximately a one-hit process for the "aggregation-reactive" molecule. The concentration of unchanged material has been calculated from the ultracentrifuge diagrams. Comparisons with osmotic pressure data from Rideal and Roberts (13) indicate that one "activated" molecule reacts with one unactivated molecule.

The above aggregation mechanism is not temperature-dependent and seems to take place very rapidly. Other aggregation reactions exist also where irreversible and constant changes in the surface structure probably are the cause. Increased aggregation when irradiated solutions are brought to pH's close to the isoelectric point and lowering of the coagulation temperature are thus explainable.

Aggregation occurs both in oxygen and nitrogen atmosphere but is more pronounced in the latter.

Aggregation occurs more easily near the isoelectric point.

The effect of substances such as sodium caprylate and perfluoro-octanoic acid on the aggregation has been investigated.

No clear-cut evidence of an extensive splitting has been found. It is not known whether the peptide bond is involved at all. Possibly this may be the case when irradiation has proceeded for a very long time under conditions where aggregation is negligible.

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